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Effect of the Methanol Leaf Extract of *Azadirachta indica* on Lead Induced Micronuclei Formation, A Marker of Genotoxicity

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Abstract

The toxic effect of lead (Pb) on the haematology and DNA of rats has been reported to result in alteration in blood cell counts and mutations that may cause cancers. In this study, the ameliorative effect of the methanol extract of *Azadirachta indica* leaf (MEAI) on lead induced haematological dyscrasias and micronuclei formation was studied on male Wistar rats. Rats of average weight 100g to 150g, were randomly divided into 7 groups of 10 rats each. Group A rats served as the control group while groups B-D rats were exposed to 0.1% lead acetate and groups E-G rats exposed to 0.2% lead acetate. Groups C and F rats were treated with 100mg/kg MEAI while groups D and G rats were treated with 200mg/kg MEAI. Standard assays were employed in the assessment of haematological changes while the micronuclei *in vivo* assay technique was used to test for genotoxicity. There was a significant ($p<0.05$) decrease in PCV and haemoglobin levels, while there was a significant ($p<0.05$) increase in monocyte counts in group B compared to the control animals (group A). These parameters were significantly ($p<0.05$) reversed at treatment with 100 (group C) and 200mg/ml (group D) MEAI. There was also significant ($p<0.05$) and dose dependent reduction in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) observed in the MEAI treated groups (Groups C, D, E and F) compared to the lead induced groups (Groups B & E). This study demonstrates MEAI as a potential drug candidate in the amelioration of lead acetate induced haematological dyscrasias amd genotoxicity.

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INTRODUCTION

Lead is a heavy metal extensively used in manufacturing industries and present in the environment in significant quantities. Despite its high level of toxicity, it is commonly used due to its malleability, poor conductivity, high density as well as its softness (Hemmaphan and Bordeerat, 2022). These, in addition to its high solubility in aqueous substances and easy absorption, results in its continuous and consistent presence in the environment thereby incessantly posing danger to the environment and its occupants (Gidlow, 2015). Depending on the level and duration of exposure, the health problems caused by lead toxicity may vary in severity. The accumulation of lead in body organs and systems causes organ and systemic damages even to DNA levels. High levels of lead in the blood of ducks have been correlated with low haematocrit levels and abnormalities in erythrocytes (Ferreyra *et al.*, 2015), Abnormalities in monocytes exposed to lead has also been reported (Metryka *et al.*, 2021). This adverse effect of lead on PCV and Hb levels has also been reported in children (Kuang *et al.*, 2020).

Studies have shown that lead exposure increases the incidence of signs of DNA damage such as micronuclei formation, chromosomal aberration, DNA breakage, inhibition of DNA synthesis, amongst others (Ibrahem*et al.*, 2020). Lead induced genotoxicity refers to the propensity of lead in causing damage to the genetic material (DNA) of an individual, which might lead to mutations and cancers. The mechanism by which this occurs, though not yet well known, has been linked to the damage of the DNA by lead induced oxidative stress, the inhibition of DNA repair by lead as well as the alteration of gene expression, which may result in genomic instability and the development of cancer (Chinde*et al.*, 2014; Shah *et al.*, 2016). Furthermore, some of these damages may affect the replication and transcription of genes, thereby causing generational mutations (Hemmaphan and Bordeerat, 2022). 8-OHdG (8-hydroxy-2'-deoxyguanosine) is a major form of oxidative lesion in DNAs and has been shown to be a strong biomarker of lead-induced oxidative stress (Valavanidis*et al.*, 2009, Ibrahem*et al.*, 2020). Lead has also been shown to substitute calcium and zinc in DNA-repair enzymes and thus inhibit DNA repair machinery, an essential protective mechanism in toxin exposed DNA (García-Lestón, *et al.*, 2010).

There is constant exposure of living things to an extensive range of substances that may impair their DNA. The DNA is endowed with strong repair and damage-bypass systems, which reliably safeguard the DNA, either by eradicating or by tolerating the damage, to ensure a general survival. Disruption or dysregulation of this DNA repair mechanisms results in genome instability, subsequently leading to diverse malignancies and disturbance of cellular metabolic equilibrium (Chatterjee and Walker, 2017).

Azadirachta indica (AI) (neem), a tree native to Asia and Africa, is well known for its effective traditional treatment of several medical conditions (Islas *et al.*, 2020). It is known to possess abundant bioactive compounds which have been demonstrated to mitigate a number of disease conditions, some of which are hypertension, diabetes and cancer. AI leaf extracts have also been shown

to own high levels of flavonoids, alkaloids, saponins, tannins, glycosides as well as to have developed a specific set of glycoproteins known as neem leaf glycoprotein which possess immune-modulatory activity, thus restricting tumour growth (Dash *et al.,* 2017). *Azadirachta indica* contains limonoids, a chemical that has been researched to possess potent antioxidant and anti-radical scavenging activities, amongst others (Priyadarsin*et al.,* 2009). It has been reported to improve weight gain, Hb and PCV levels in broilers on neem included diet (Paul *et al.,* 2020). Crude terpenoid from neem leaf has also been reported to decrease monocyte counts (Gupta and Chaphalkar, 2016).

Oyagbemi et al. (2017) reported AI to be more genoprotective than Vitamin E. Indeed, the protective effect of neem against lead induced genotoxicity in *Allium cepa* has been documented by Shalini *et al.* (2018). Furthermore, a major limonoid in AI, nimbolide, has been found to possess an extensive field of pharmacological properties, including anticancer activity. It has been documented to avert continuous proliferation, angiogenesis, invasion, apoptosis evasion and metastasis, all of which are hallmarks of cancer (Nagini*et al.,* 2021). The medicinal effects of AI leaf have been linked to cellular and molecular mechanisms, which infer its ability to modulate genetic information and cellular signaling pathways (Omobowale*et al.,* 2016, Patel *et al.,* 2016). This study is designed to investigate the haematological and anti-genotoxic effect of MEAI in lead exposed rats.

MATERIALS AND METHODS

Experimental Animals

70 male wistar rats were randomly divided into 7 groups of 10 rats each. They were allowed to acclimatize for 2 weeks and fed *ad-libitum* with pelletized rat feed and clean drinking water during this period. Group A, the control group, was not exposed to either lead or MEAI. Groups B, C and D were given 0.1% Pb acetate in water for 6 weeks. At the end of 6 weeks, groups C and D were given 100mg/kg and 200mg/kg MEAI respectively for another 6 weeks while group B was given clean drinking water for recovery purposes. Groups E, F and G were given 0.2% Pb acetate in water for 6 weeks at the end of which groups F and G were given 100mg/kg and 200mg/kg MEAI respectively for another 6 weeks while group E was given clean drinking water. All the rats were kept in the same environment and given the same feed throughout the 12-week period of the experiment.

Source of Lead and MEAI

Lead (ll) acetate trihydrate ($Pb(CH_3COO)_2.3H_2O$, M=379.33) L/no: XK13-011-00005 CAS(6080-56-4) was purchased from a leading chemical supplier in Ibadan. *Azadirachta indica* leaves were obtained from a Neem tree at Oyo town, air dried in a cool room and blended into a finer form. They were de-fatted by soaking in n-hexane for 24 hours and then in 100% methanol for 72 hours. The extract was then concentrated in a rotary evaporator and dried.

Administration of Lead and MEAI

Lead acetate salt was weighed and dissolved in clean water at a concentration of 1 mg/ml

and 2 mg/ml. It was administered to the rats ad-lib. The dried MEAI was weighed and dissolved in Corn oil at a stock concentration of 80mg/ml. MEAI was administered to the rats according to their average weight.

Blood Collection and Harvesting of Organs.

Five milliliters of blood were collected at the medial canthus of each rat through the orbital vein with the use of capillary tubes. From each rat, the blood samples were collected into 2 EDTA bottles. The first set of collected blood was spun at 4000 rev/min for 10mins and the plasma decanted. It was then washed with Normal saline about thrice and later lysed with distilled water. Both the lysed RBCs and the plasma were kept at -4^{o}C.

Haematology

The microhaematocrit, cyanmethaemoglobin and microscopic methods were utilized in determining the PCV, Hb concentration and monocyte count

Genotoxicity

This was carried out with the use of the *in-vivo* micronucleus assay technique. The proximal extremities of the femur were cautiously removed with a pair of scissors till a slight opening to the marrow became discernable. The femur was submerged in fetal calf serum and the marrow was flushed out mildly by aspiration and flushing on the glass slides. The marrow suspension was placed on one end of a slide and spread by dragging a polished glass cover held at an angle of 45^{o}C. The slides were fixed in methanol for 3-5 minutes, permitted to dry for one day, further stained with May-Gruenwald stain and subsequently with 5% diluted Giemsa solution for a minimum of 30 minutes. Following this, slides were rinsed in phosphate buffer for approximately 30 seconds, then in distilled water and air dried. The dried stained slides were mounted in DPX (DNA-protein crosslinks) with coverslips and observed for the presence of micro-nucleated polychromatic erythrocytes (MNPCE), under the microscope at ×100 magnification, using oil immersion. A tally counter was utilized for scoring (Schmid, 1975)

Data Analysis

Data collected were analyzed using the student's T test (Elston and Johnson, 2008) as well as the one-way Anova at the level of 5% significance and were presented as Mean ± standard deviation, using the PRISM software package (Version 5.0)

RESULTS

Hematology

The effect of Pb and MEAI on haematology is represented in Figure 1. There was a significant ($P < 0.05$) reduction in PCV and Hb concentration at 0.1% lead exposure (Group B) compared with the control (Group A) as well as a significant ($P < 0.05$) increase in these parameters at 100 mg/ml MEAI (Group C) compared to the 0.1% lead exposure (Group B). There is however no significant reduction in PCV and Hb concentration at 0.2% lead exposure (Group E) compared to the controal (Group A). MEAI at 100 and 200 mg/ml caused an insignificant increase in Groups F & G compared to the toxicant group (Group E). There was a significant increase in monocyte

count in group B (0.1% Pb) compared to the control (Group A), the 100 and the 200 mg/ml MEAI treated groups (Groups C & D).

Genotoxicity

Figure 2 shows the precipitation of genotoxicity in the groups. The frequency of micronuclei was significantly ($P < 0.05$) increased in groups B (0.1% Pb) and E (0.2% Pb) compared to the control (Group A), while there was significant ($P < 0.05$) and dose dependent reduction in the frequency of micronuclei in the MEAI treated groups (Groups C, D, F & G) compared with the respective toxicant groups (Groups B and E

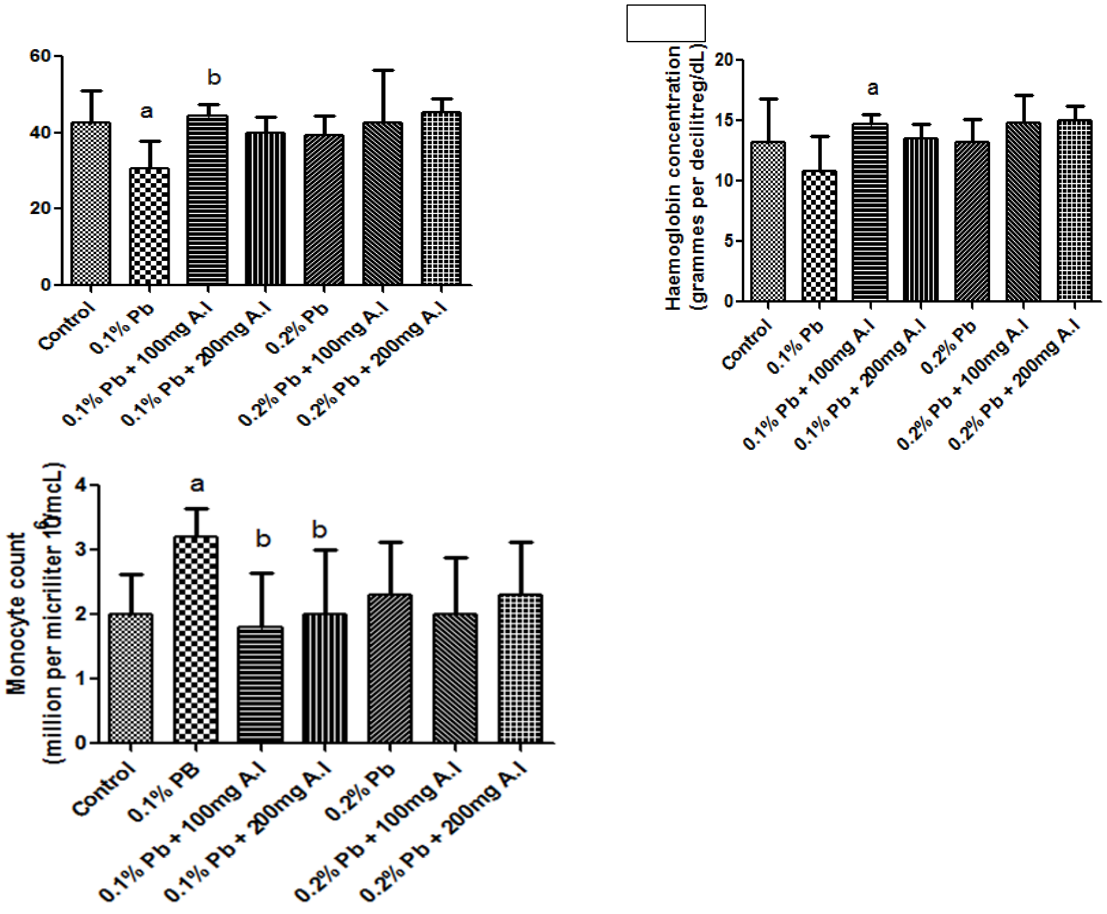

Figure 1: Figure Showing Packed Cell Volume (PCV), Haemoglobin (Hb) Concentration and Monocyte Count. Superscript a indicates significantly different from control, superscript b: significantly different from 0.1% Pb

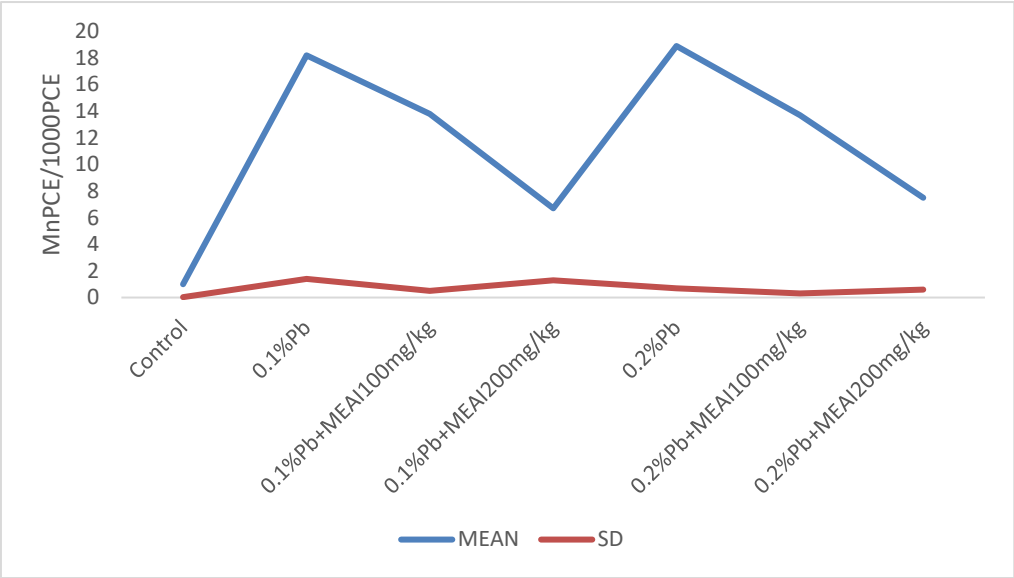

Figure 2: Graph showing Mononuclei Frequency. Unit of mononuclei frequency is expressed in MnPCE/1000PCE. Pb (Lead acetate), MEAI (Methanol extract of *Azadirachta indica* leaf)

DISCUSSION

A major hallmark of lead toxicity is anaemia. Significant correlation has been established between lead levels in the blood and Haemoglobin (Hb) as well as Packed Cell Volume (PCV) levels (Moayedi *et al.*, 2008; Kim & Lee, 2013; Mazumdar and Goswami, 2014) while Stoleski *et al.* (2008) reported no particular correlation between lead levels in blood and PCV but only with Hb. This study demonstrated a significant ($P<0.05$) reduction in PCV and Hb levels of most of the lead exposed rats. Earlier reports by Gidlow (2015), had established hypochromic, normocytic, or microcytic anemia with reticulocytosis in chronic lead overdose. The reduction in Hb concentration in lead (Pb) toxicity has been proposed to be due to the binding of more than 90% of Pb in the blood to Red Blood Cells (RBC) (Mrugesh *et al.*, 2011). The lead-induced hypochromic anaemia observed in this study can be linked with oxidative stress, damage to the bone marrow, kidneys and body organs essential for blood production, impairment of Hb synthesis pathways or prevention of key enzymes in Hb synthesis (Assi *et al.*, 2016). There is also a likelihood of lead interfering with energy and transport systems, which reduces the length of time that the cells

survive (Mazumdar and Goswami, 2014; Kshirsagar *et al.,* 2015). Lead toxicity may also result in improper erythropoietin production, inadequate red blood cell maturity, and ultimately anemia (Kshirsagar *et al.,* 2015). Furthermore, monocytosis has been linked with systemic and chronic inflammation, acute stress, autoimmune disorders and haemolytic anaemia (Lynch et al., 2018). Lead-induced increase in monocytes had earlier been reported by Ilesanmi et al (2022), although there are contradictory findings on the effect of lead on monocyte count in other studies. In this study, significant ($p<0.05$) increase was observed at 0.1% lead exposure which may be as a result of lead, inducing stress, inflammation or immune disorder.

In concert with our result, Kayath *et al.* (2022) reported a significant increase in haematological parameters, including PCV and Hb levels, of neem supplemented kuroiler chicken. This was reiterated by Musa *et al.* (2022) but had been earlier countered by Ikwuka *et al.* (2020) who reported not so much significance in the PCV and Hb levels of neem fed animals. Gangar *et al.* (2010) also reported a reversal of reduced Hb concentrations induced by benzo(a)pyrene in AI (100mg/kg) treated rats. Reduction in previously increased monocyte count is indicative of an attenuation of inflammatory response and possible restoration of the immune status of the animal. The immunosuppressive and antiinflammatory effects of extracts from neem leaf have been proposed by Gupta and Chaphalkhar (2016)

In vitro and *in vivo* test systems have demonstrated that lead acetate is a powerful inducer of micronuclei (Abd El-Monem, 2012). In this study, lead acetate precipitated micronuclei formation at both 0.1% and 0.2% exposure. Pb has the ability to cause single-strand DNA breaks, probably via displacing metal binding sites. It has been demonstrated to cause toxic effects and to compete with calcium for synaptosome entrance (Virgolini and Aschner, 2021). There can be indirect induction of genotoxicity. This can be by the interaction of lead with DNA related proteins which are involved in transcription, replication or repair by inhibiting enzyme activity, modifying DNA pathways or by ROS oxidation. Hartwig *et al.* (1990) demonstrated an indirect mechanism, showing that lead ions obstruct the processing of UV-induced DNA damage at all endpoints studied. Pb prevents DNA strand breaks from being repaired after exposure to UV light and increase the frequency of UV-induced mutations and sister chromatid exchanges, both of which point to a reduction in DNA repair. Exposure of *Clarias gariepinus* to lead nitrate for 2 and 4 weeks significantly increased the percentage of poikilocytosis, micronuclei, and apoptotic cells as well as comet tail length and olive tail moment compared to control catfish. These cytotoxic and genetic alterations were lessened by dietary supplements, and this improvement was concentration- and time-dependent (Hamed *et al.,* 2019).

The genoprotective activity of *Azadirachta indica* has been earlier reported by other researchers, including Oyagbemi *et al.* (2017). In this study, the reduction in the frequency of micronucleated cells of the treated groups is indicative of the ameliorative effect of MEAI in lead-induced

genotoxicity in rats. Although MEAI significantly ($P<0.05$) reduced the frequency the micronucleated cells, it did not restore it back to normalcy, thus corroborating the findings of Oyagbemi *et al.* (2017). The reversal of genotoxicity may be linked to the antioxidant and phytochemical content of MEAI. It also reveals its potential to enhance the DNA repair mechanism. Interestingly, the genotoxic effect of the aqueous leaf extract of *Azadirachta indica* on *Allium cepa* bulbs has been earlier reported by Akaneme and Amaefule (2012), thus suggesting the need for proper dosage and duration in the use of MEAI. An LD_{50} of 31.26g/kg has however been proposed by Akin-Osanaiye *et al.* (2013) while toxicity tests have shown that at a dose of up to 1200mg/kg, A.I does not have embryotoxic nor genotoxic effects in Wistar rats (Ramalho *et al.,* 2023). This gives credence to the safety of the dosage of 0.1 and 0.2 g/kg utilized in this present study.

Furthermore, carcinogenic substances have been known to alter hematological parameters (Gangar *et al.*, 2010), such as was seen with Pb in this study. Neem leaf extract has been shown to stimulate hematological systems, as demonstrated by an increase in hemoglobin percentage, total count of RBC, WBC, and platelets. This is in addition to its immunostimulatory and the growth restrictive activity of murine cancer (Haque *et al.*, 2006). MEAI stimulated the Pb-depressed PCV and Hb levels, reduced the excessive monocyte levels and reversed the Pb-induced genotoxicity, thus pointing to a strong correlation between the genoprotective and haematological effects of this extract, as well as a possible anticarcinogenic potential.

CONCLUSION

The potential of MEAI as a drug candidate in the amelioration of lead acetate anaemia and monocytosis as well as the precipitation of micronuclei has been demonstrated in this study. This has been linked to its antioxidant and immunostimulatory properties of *Azadirachta indica* leaf. Investigations should be carried out on individuals to check their lead levels. Diagnostic tools that are affordable and readily accessible are required. There is a need to utilize *Azadirachta indica* as a possible supplement for regular usage to combat possible genotoxic effects of environmental toxins. Literature is replete with information on the properties and constituents of *Azadirachta indica.* These should be further investigated and isolated to formulate blood enhancing components as well as targeted against compounds that induce genotoxicity.

AUTHORS CONTRIBUTION

The supervision and conceptualization of this study was by T.O. Omobowale; project administration was by A.O. Oyagbemi and A.A. Adedapo, data curation and methodology was carried out by O.O. Ola-Davies and T.E AdeyeOluwa; research work, data generation and analysis as well as writing up of article done by T.E. AdeyeOluwa

ETHICAL APPROVAL

This study was approved by the UI-ACUREC and given registration number NHREC/UIACUREC/05/12/2022A

CONFLICT OF INTEREST

The authors declare that they have no known competing interests or personal relationships

that could have appeared to influence the work reported in this paper

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