Ameliorative Effects of Moringa (*Moringa Oleifera* Lam.) Leaves Extract on Lead-Induced Oxidative Stress, Hepcidin and δ-Alad Levels in Rat's Blood

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ABSTRACT

Introduction: Lead (Pb) is a toxic heavy metal that cause a lot health problem. Blood, especially hemoglobin and erythrocyte, is the main target of lead poisoning. Literatures explain that moringa has phytochemical contents to reduce heavy metal poisoning. This study aimed to examine ameliorative effects of moringa leaves extract on oxidative stress, hepcidin increasement and δ-alad level decline induced by lead poisoning in the blood of rat model. **Methods:** This study was completely randomized posttest-control group design. Forty-eight males *Rattus norvegicus* Wistar strain rat were divided into 4 groups. The control group or G0 (given Pb orally doses of 750 mg/kgBW/day for 7 days and was not given 50% ethanol extract of moringa leaves/MLEE). Three treatment groups (G1, G2 and G3), all were given Pb at a dose of 750 mg/kgBW/day orally for 7 days, followed by administration of MLEE for 14 days at a dose of 250 mg/kgBW/day, 500 mg/kgBW/day and 1,000 mg/kg/day orally, respectively. Blood samples were taken one day after 14 days of MLEE treatment. Pb levels was examined by AAS and δ-ALAD levels, GSH levels, MDA levels and hepcidin levels examined by ELISA. **Results:** MLEE doses 1,000 mg/kgBW/day for 14 days increased δ-ALAD levels, GSH levels, hepcidin levels and reduce MDA levels significantly compared to the control group. **Conclusion:** Moringa leaves ameliorate lead-induced poisoning by reducing oxidative stress, declining hepcidin, and increasing δ-ALAD in the blood of male *Rattus norvegicus* Wistar strains rats. Moringa leaves is beneficial to address Pb poisoning in the blood through antioxidants, anti-inflammation, and improving δ-ALAD level in the blood of Wistar strain rats.

Key words: Blood, δ-ALAD, Hepcidin, Lead poisoning, Moringa, Oxidative stress.

INTRODUCTION

Lead (Pb) is a heavy metal that causes a lot of health problems. Pb exposure in the general population mainly occurs *via* ingestion and inhalation. The normal threshold for Pb levels is 10 g/dL. In Indonesia, several studies have shown that the prevalence of blood Pb levels >10 g/dL in workers is very high, ranging from 30% to 100%.[1,2] The 100% prevalence was found in battery recycling and metal foundry industry workers.[3,4]

After entering the digestive tract, Pb will be absorbed in the intestine. Pb absorption, will cause an increase in Pb levels in the blood. Hematology system, is the first system that is affected by adverse effect of Pb exposure. The most susceptible are hemoglobin and erythrocyte, as after being absorbed, 99% of Pb is bound to erythrocytes and 85% is bond to haem. Lead-induced blood poisoning in haem and erythrocyte mainly occurs through two main mechanisms, namely impaired heme biosynthesis and increased erythrocyte damage rates.[5,6] Pb inhibits hemoglobin synthesis through delta aminolevulinic acid dehydratase (δ-ALAD) and ferrochelatase enzymes inhibition, which are essential for heme synthesis. Both enzymes are susceptible to Pb because they contain a sulfhydryl or thiol group that can bind Pb well.[7,8] Erythrocyte damage occurs because Pb causes the erythrocyte cell membrane to be fragile, so that the life span of erythrocytes is reduced to only one-sixth of normal erythrocyte cells.[6,9-12]

Erythrocyte damage due to Pb exposure occurs mainly through oxidative stress and inflammation.[6,13,14] Pb binds covalently (Pb^{2+}) with sulfhydryl (SH) or thiol groups of the antioxidant defense system, resulting in an increase of oxidative stress.[15,16] Oxidative stress also can occur because the inhibition of δ-ALAD by Pb increases the levels of delta aminolevulinic acid (δ-ALA), which triggers the formation of reactive oxygen species (ROS). ROS and the binding of the SH group of GSH by Pb results in a decrease in GSH levels which act as antioxidants, thereby causing oxidative stress, which in turn causes lipid peroxidation which is characterized by an increase in malondialdehyde (MDA) levels.[14] Pb toxicity through the inflammatory pathway mainly occurs with an increase in specific inflammatory mediators, especially cytokines. Inflammation will cause an increase in hepcidin levels. Hepcidin acts as a negative regulator of intestinal iron absorption and release by macrophages. Hepcidin binds to the ferroportin receptor and causes the internalization and degradation of ferroportin and iron retention in enterocytes. As a result, absorption and mobilization of iron stores from the liver and macrophages decreases, iron remains bound to ferritin, causing a decrease in serum ferritin levels.[17-19] Inhibition of haem synthesis and erythrocyte damage due to chronic Pb exposure results in irreversible anemia. Therefore, prevention of irreversible anemia due to Pb exposure needs to be done as soon as possible after Pb exposure can be detected and irreversible anemia has not occurred.

Indonesian people frequently use natural plants to combat poisoning. In Indonesia, the use of medicinal plants has been carried out for generations in various regions and is believed to be safer than the use of synthetic chemical drugs. One of natural plants in Indonesia that has the potential to ameliorate lead exposure is moringa (*Moringa oleifera* Lam.). Moringa is a very well-known plant and is widely used throughout the world for its nutritional and medicinal properties.[20] Moringa leaves rich of biologically active phytoconstituents such as flavonoids, polyphenols, alkaloids, carotenoids, glycosides in addition to the highest content of amino acids, minerals and vitamins, are traditionally and scientifically used in the treatment of various diseases, nutritional deficiencies and health conditions. Flavonoids have been shown to have a protective effect against chronic diseases associated with oxidative stress. The main flavonoids of Moringa leaves are myrecytin, quercetin and kaempferol. Quercetin also has properties as a heavy metal chelator. Phenolic acid has antioxidant, anti-inflammatory, antimutagenic, and anticancer properties. Gallic acid is the most abundant phenolic acid in Moringa leaves.[21,22] This study aimed to examine ameliorative effects of moringa leaves extract on oxidative stress, hepcidin increasement and δ-alad level decline induced by lead poisoning in the blood of rat model.

METHODS

Ethical clearance

This study was endorsed by Ethical Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia with ethical approval No. 2.KE.037.04.2020.

Moringa leaves extraction

Fresh Moringa leaves were collected from a moringa garden in Yogyakarta, Indonesia. Fresh moringa leaves were washed, and then shaded with cloth and dried under sun shine until dry. About 1 kg dried moringa leaves were extracted using 50% ethanol in an extractor, and were macerated for 24 hours. Maceration was conducted three times. Liquid extract obtained from the maceration was freeze dried to obtain solid extract. The detailed method was as described in detail by Mahdi *et al.* (2016). The solid extract was put in the brown bottle and kept in the refrigerator.[23]

Study design

This study was completely randomized posttest-control group design.

Animal and treatment

Forty-eight local strain adults male Wistar rats weighing 150-200 grams, 2,5-3-month-old were used in this study. Rats were obtained from Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Yogyakarta, Indonesia. Rats were acclimated to the laboratory room environment for 7 days at room temperature (20-24^{0}C) and at humidity level of 40-70%. They were fed with standard commercial rat pellets and tap water. The rats were divided into 4 group randomly, with 12 rats per group.

The groups and the treatment of each group were as follows:

Control group (G0): The rats received Pb-acetate 700 mg/kgBW per day orally for 7 days, followed by administration of aqua for 14 days.

Experiment group 1 (G1): The rats received Pb-acetate 700 mg/kgBW per day orally for 7 days, followed by administration of 1.000 mg 50% ethanol extract of moringa leaves orally for 14 days.

Experiment group 2 (G2): The rats received Pb-acetate 700 mg/kgBW per day orally for 7 days, followed by administration of 500 mg 50% ethanol extract of moringa leaves orally for 14 days.

Experiment group 3 (G3): The rats received Pb-acetate 700 mg/kgBW per day orally for 7 days, followed by administration of 250 mg 50% ethanol extract of moringa leaves orally for 14 days.

Administration of Pb-acetate and 50% ethanol extract of moringa leaves to all rats were conducted in the morning, between 7-8 am, local time.

Blood collection samples

Blood samples from experimental animals in this study were taken the day after 14 days of being given Moringa leaf powder. Blood samples were taken intracardiac under ketamine-xylazine-acepromazine anesthetic.

GSH, MDA, hepcidin and δ-ALAD levels

GSH and MDA as indicators of oxidative stress, hepcidin as indicator for inflammation, and δ-ALAD levels were measured by enzyme-linked immunosorbent assay (ELISA). ELISA kits for GSH, MDA, hepcidin and δ-ALAD levels measurements were purchased from Bioassay Technology Laboratory, Shanghai, China with catalogue no. E1101Ra for GSH, E0156Ra for MDA, E0597Ra for hepcidin, and E1184 for δ-ALAD. The measurement procedures were following the catalogue available in the ELISA kits.

Statistical analysis

Data are presented the mean ± standard deviation values. One-way analysis of variance (ANOVA) at the confidence level of $p<0.05$ followed by Tukey HSD post hoc test were then used to compare other groups. If the data was not normally distributed, Kruskal Wallis test with Mann Whitney U post hoc test were employed.

RESULTS

GSH levels in this study can be seen in Table 1. The highest average GSH level was found in the G1 group, while the lowest was found in the control group (G0). In general, the average GSH levels in all treatment groups were higher than that in the control group. The increased of GSH levels indicated that oxidative stress could be reduced. Statistical analysis showed that there was a significant difference in GSH levels between the control group and the G1 group (p=0.001), while with the G2 and G3 groups there were no significant difference in GSH levels, with p-values of 0.992 and 0.998, respectively.

This study found that the administration of MLEE could reduce plasma MDA levels in all treatment groups (G1-G3) compared to control group (G0). The decrease in plasma MDA levels indicated that lipid peroxidation caused by oxidative stress due to Pb exposure could be minimized by administering MLEE (Table 2). As the data distribution for MDA level was not normally distributed, statistical analysis used were Kruskal Wallis and Mann Whitney U test for post hoc test. Statistical analysis showed that these were significant difference in MDA levels between the control group and the G1 group (p=0.000) and G2 (p=0,000), while with the G3 group there was no significant difference in MDA levels, with p-values of 0.775.

The highest mean hepcidin level was found in the control group, whereas the lowest was in the P1 group. The administration of MLEE at a dose of 1,000 mg/kg BW (Group G1) had the lowest hepcidin content. The decreased of hepcidin level means that the inflammatory process induced by lead that triggers an increase of hepcidin levels can be minimized (Table 3).

As the data distribution for hepcidin level was not normally distributed, statistical analysis used were Kruskal Wallis and Mann Whitney U test for post hoc test. Statistical analysis showed that these were significant difference in MDA levels between the control group and all treatment groups (G1, G2, and G3), with p-values of 0.000 in all groups.

Table 1: GSH levels after treatment in all groups and statistical analysis results.

Group	N	GSH level (mg/L)	One way ANOVA		Tukey HSD post hoc test
			F value	p-value	
G0	12	272.47 ± 53.89			-
G1	12	384.77 ± 78.71			0.001
G2	12	280.16 ± 68.54	8.083	0.000	0.992
G3	12	277.12 ± 60.33			0.998

Table 2: MDA levels after treatment in all groups.

Group	N	MDA level (nmol/ml)	Kruskal Wallis test		Mann Whitney U post hoc test
			X^2	p-value	p-value
G0	12	3.09 ± 0.56	30.227	0.000	-
G1	12	1.89 ± 0.25			0.000
G2	12	1.80 ± 0.24			0.000
G3	12	4.74 ± 3.66			0.775

Table 3: Hepcidin levels after treatment in all groups.

Group	N	Hepcidin level (ng/ml)	Kruskal Wallis test		Mann Whitney U post hoc test
			X^2	p-value	p-value
G0	12	666.10 ± 129.88	30.227	0.000	-
G1	12	287.22 ± 57.34			0.000
G2	12	289.05 ± 47.85			0.000
G3	12	300.49 ± 47.30			0.000

Table 4: Results of δ-ALAD level measurement after treatment.

Group	N	δ-ALAD level (ng/ml)	Kruskal Wallis test		Mann Whitney U post hoc test
			X^2	p-value	p-value
G0	12	8.53 ± 2.26	26.919	0.000	-
G1	12	24.77 ± 9.21			0.000
G2	12	10.47 ± 2.60			0.143
G3	12	9.56 ± 2.27			0.514

The highest mean δ-ALAD level was found in the G1 group, while the lowest average δ-ALAD level was found in the control group (Table 4). Based on the data in Table 4, it is known that in all treatment groups, the average levels of δ-ALAD were higher than the control group, but the highest average was found in the P1 treatment group, which were treated with MLEE at a dose of 1,000 mg/kg BW. Increased levels of δ-ALAD indicate that the binding of Pb to δ-ALAD can be reduced, so that the impact of Pb on the inhibition of heme formation could be minimized.

As the data distribution for δ-ALAD level was not normally distributed, statistical analysis used were Kruskal Wallis and Mann Whitney U test for post hoc test. Statistical analysis showed that there was significant difference in δ-ALAD levels between the control group and G1 group (p=0.000), whereas between G2 dan G3 no significant different were found, with p= 0.143 and p=0,514, respectively (Table 4).

DISCUSSION

The result showed that there was a significant increase in GSH levels in G1, which was treated by MLEE doses 1,000 mg/kgBW/day orally for 14 days. This result indicates that the administration of 50% ethanol extract of Moringa leaves can improve oxidative stress caused by Pb toxicity. The main mechanism of Pb toxicity is through oxidative stress. In lead poisoning, the formation of free radicals exceeds the ability of the body's antioxidant system to detoxify free radicals, resulting in the accumulation of free radicals that cause cell damage.[24] Oxidative stress occurs through enzymatic and non-enzymatic pathways. Pb ions have been shown to be associated with increased ROS formation, and are able to interfere with antioxidant defenses, including antioxidant

enzymes and non-enzymatic antioxidants.[11] Pb also causes oxidative stress through non-enzymatic pathways. Pb inhibits nonenzymatic molecules such as glutathione (GSH) or by replacing zinc ions which act as important cofactors at their catalytic sites and inactivating them.[25]

Lead is believed to deplete antioxidant defenses, including reduced glutathione (GSH) reserves. GSH functions as one of the most important antioxidants in the human body because it is a tripeptide with asulfhydryl group, which scavenging ROS and acts as a cofactor for antioxidant enzymes, such as glutathione peroxidase (GPx), catalase (CAT) and Glutathione-S-transferase (GST). As a result, oxidative stress conditions are increased when lead ions are present in the biological system.[17]

In this study, administration of MLEE was proven to increase GSH levels, thereby improving oxidative stress caused by Pb toxicity. Moringa leaves (*Moringa oleifera* Lam.), which are widely used by the community to treat poisoning from the body, are a good source of flavonoids. The main flavonoids in Moringa leaves are myrecytin, quercetin and kaempferol. Flavonoids, which are synthesized by plants in response to microbial infection, have a benzo-pyrone ring as a general structure, and have been shown to protect against diseases associated with oxidative stress.[22] Lamidi *et al* (2020) stated that flavonoids can bind Pb through the formation of complexes between flavonoids and Pb.[26]

From the results of the study, it was found that the administration of MLEE at a dose of 1,000 mg/kg BW (G1 group) and 500 mg/kgBW (G2 group) could significantly reduce plasma MDA levels compared to the control group. The decrease in plasma MDA levels indicated that lipid

peroxidation caused by oxidative stress due to Pb exposure could be minimized by administering 50% ethanol extract of Moringa leaves.

Decreased GSH levels and decreased SOD, CAT and GPx activity in lead-exposed erythrocytes may play a role in increased membrane lipid peroxidation. Lead can be directly attached to the cell membrane, thereby increasing the sensitivity of the membrane to the lipid peroxidation process. Malondialdehyde (MDA) is the end product in the lipid peroxidation process, and can be used as a good biomarker for membrane lipid peroxidation.[16]

In this study it was proven that the administration of MLEE can reduce plasma MDA levels, which is the end product of lipid peroxidation. The antioxidant effect of MLEE is mainly due to its high flavonoid content. Flavonoid compounds are polyphenolic compounds consisting of 15 carbon atoms configured C6-C3-C6, which means that the carbon skeleton consists of two C6 groups (substituted benzene rings) which are then joined by a three-carbon aliphatic chain.[25] Flavonoids also include secondary metabolites of polyphenols. Flavonoids are found widely in plants and food, and have various bioactive effects including anti-viral, anti-inflammatory, antidiabetic, cardioprotective, antiaging, anti-cancer, and antioxidant.[27]

Flavonoids have the ability to prevent injury caused by free radicals and can stabilize ROS which have the ability to bind to free radicals that cause degenerative diseases, by deactivating free radicals.[28] Flavonoids act as antioxidants because they have hydroxyl groups that can donate hydrogen atoms to free radical compounds and stabilize reactive oxygen compounds (ROS) and have hydroxyl ketone groups that have a role as metal chelators that function as catalysts in lipid peroxidation.[29] A study conducted by Adhikari *et al* (2018) on Swiss albino rats concluded that the antioxidant activity (2,2-diphenyl-1-picrylhydrazyl assay) of the Pb–morin complex, a type of flavonoid, was more sustainable than that of Pb-free morin.[30]

This study established that the administration of MLEE doses 1.000 mg/kgBW can reduce hepcidin levels significantly compared to the control group in G1 group. This shows that the inflammatory process or inflammation that triggers an increase in hepcidin levels can be minimized so that hepcidin levels decrease. Hepcidin is an antimicrobial peptide hormone synthesized by the liver in response to inflammatory stimuli and iron overload. Hepcidin is a major regulator of iron homeostasis, where its synthesis is mainly controlled by bone marrow erythropoiesis activity, iron storage, and the presence of inflammation in the body (Purwanto, 2018).

In this study, there was a significant decrease in hepcidin levels after administration of 1,000 mg/kgBB MLEE. This proves that 50% ethanol extract of Moringa leaves can suppress the inflammatory process caused by exposure to Pb. This is in accordance with the research results of Simorangkir *et al* (2020).[31] They, in their research, proved that the administration of ethanol extract of Moringa leaves had an anti-inflammatory effect on inflamed male Wistar strain rats. The anti-inflammatory effect is thought to be due to the activity of secondary metabolites contained in the ethanol extract of Moringa leaves, namely flavonoids.[32]

Inflammation on lead exposure occurs because oxidative stress causes the release of pro-inflammatory interleukins, including IL-6, which triggers hepcidin production. As previously explained, the MLEE contains high flavonoids. Flavonoids act as antioxidants because they have hydroxyl groups that can donate hydrogen atoms to free radical compounds and stabilize reactive oxygen compounds (ROS) and have hydroxyl ketone groups that have a role as metal chelators that function as catalysts in lipid peroxidation.[29] By decreasing oxidative stress, the inflammatory reaction can also be reduced.

The result showed that compared to the control group, δ-ALAD levels experienced a significant increase in treatment group G1, which received a dose MLEE of 1,000 mg/kgBW/day orally for 14 days. These results indicate that the administration of 50% ethanol extract of Moringa leaves can improve δ-ALAD levels, so that disturbances in hemoglobin synthesis can be minimized. The δ-ALAD enzyme is the most sensitive to Pb in the heme pathway and has a high affinity for metals. Pb binds to the SH group of the enzyme, which normally binds to zinc, preventing the binding of δ-ALA.[32,33] Winarni *et al*. (2022) and Shukla *et al* (2018) stated that lead (Pb) interferes with hemoglobin synthesis by inhibiting sulphydryl-dependent enzymes such as ferrochelatase and δ-ALAD, which are essential for heme synthesis.[6,34]

These results are similar to those of Lamidi *et al* (2020) who concluded that Daflon (a purified flavonoid) can increase δ-ALAD activity in adult male Wistar rats exposed to Pb. Moringa leaf 50% ethanol extract is rich in flavonoids.[26] Flavonoids can minimize the binding of Pb to δ-ALAD because flavonoids can bind Pb to form a complex with Pb.[30] Kim *et al* (2015) also stated that flavonoids, like other antioxidants, prevent oxidative stress by binding to heavy metal ions and preventing free radical chain reactions.[35] Polyphenols in general and flavonoids in particular, perform their antioxidant activity either by hydrogen atom transfer or by electron transfer (Nobossé et al, 2018).[36]

The limitation of this study is that antioxidants contents in the MLEE didn't calculate in determining MLEE doses. However, to date, no study was conducted to calculate flavonoid contents of the MLEE to obtain optimal doses to address Pb poisoning, especially doses that could chelating Pb in the body. Future research is needed to study about this issue.

CONCLUSION

In conclusion, moringa leaves ameliorate lead-induced poisoning by reducing oxidative stress, declining hepcidin, and increasing δ-ALAD in the blood of male *Rattus norvegicus* Wistar strains rats. Moringa leaves is beneficial to address Pb poisoning in the blood through antioxidants, anti-inflammation, and improving δ-ALAD level in the blood of Wistar strain rats.

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CONFLICTS OF INTEREST

There are no conflicts of interest in our study.

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GRAPHICAL ABSTRACT

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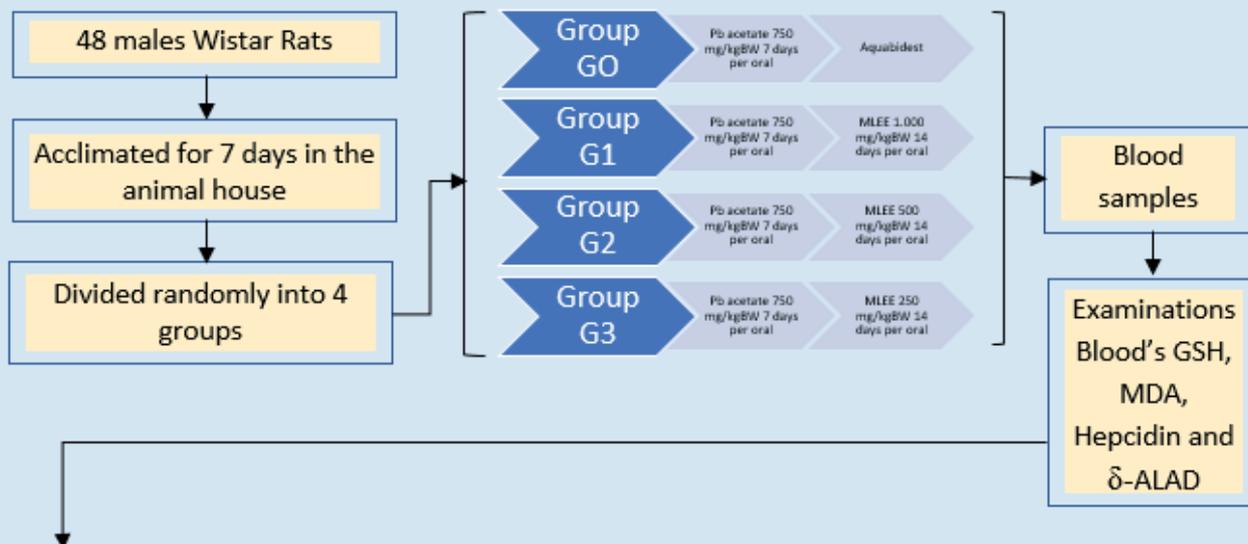
Results

Table 1. GSH levels after treatment in all groups and statistical analysis results

Group	N	GSH level (mg/L)	One way ANOVA F value	One way ANOVA p-value	Tukey HSD post hoc test
G0	12	272.47 ± 53.89			-
G1	12	384.77 ± 78.71	8.083	0.000	0.001
G2	12	280.16 ± 68.54			0.992
G3	12	277.12 ± 60.33			0.998

Table 2. MDA levels after treatment in all groups

Group	N	MDA level (nmol/ml)	Kruskal Wallis test X^2	Kruskal Wallis test p-value	Mann Whitney U post hoc test p-value
G0	12	3.09 ± 0.56	30.227	0.000	-
G1	12	1.89 ± 0.25			0.000
G2	12	1.80 ± 0.24			0.000
G3	12	4.74 ± 3.66			0.775

Table 3. Hepcidin levels after treatment in all groups

Group	N	Hepcidin level (ng/ml)	Kruskal Wallis test X^2	Kruskal Wallis test p-value	Mann Whitney U post hoc test p-value
G0	12	666.10 ± 129.88	30.227	0.000	-
G1	12	287.22 ± 57.34			0.000
G2	12	289.05 ± 47.85			0.000
G3	12	300.49 ± 47.30			0.000

Table 4. Results of δ-ALAD level measurement after treatment

Group	N	δ-ALAD level (ng/ml)	Kruskal Wallis test X^2	Kruskal Wallis test p-value	Mann Whitney U post hoc test p-value
G0	12	8.53 ± 2.26	26.919	0.000	-
G1	12	24.77 ± 9.21			0.000
G2	12	10.47 ± 2.60			0.143
G3	12	9.56 ± 2.27			0.514

Conclusion

Moringa leaves ameliorate lead-induced poisoning by reducing oxidative stress, declining hepcidin, and increasing δ-ALAD in the blood of male Rattus norvegicus Wistar strains rats. Moringa leaves is beneficial to address Pb poisoning in the blood through antioxidants, anti-inflammation, and improving δ-ALAD level in the blood of Wistar strain rats.

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