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Various parts of *Moringa oleifera* ameliorate Phenylhydrazine-Induced Spleen toxicity in Male Wistar Rats

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ABSTRACT: The toxicity of sickle cell anaemia maintenance drugs makes medicinal plants viable alternatives. The protective roles of *Moringa oleifera* (MO) parts (**B**ark, **f**lower, **l**eaf, **r**oot and **s**eed) against phenylhydrazine (PHZ) induced spleen toxicity and oxidative stress in rats were carried out. Splenomegaly was observed in anaemic rats and was reversed by *M. oleifera* parts, particularly the flower and roots. The *M. oleifera* leaves significantly reduced the elevated malondialdehyde levels to that of control rats, control rats (NOR) 4.66±0.14, anaemic rat (NEG) 10.81±0.60, hydroxyurea treated group (HU) 8.25±0.53 and *M. oleifera* leaves (MOL) 4.59±0.1. The increase in GPx and decrease in CAT observed in the anaemic rat's spleen were returned to the control value by MOL, while MOR ameliorated the GPx only. The histopathology analysis of the various treatment groups' spleen tissues corroborated the biochemical results. The results indicated that various parts of MO, particularly the leaf, attenuated the toxic effects of phenylhydrazine on the spleen. MOL can thus serve as a natural source of non-toxic sickle cell anaemia care nutraceuticals.

Keywords: *Moringa oleifera*, Anaemia, Phenylhydrazine, Splenomegaly, Nutraceuticals

Introduction

Sickle cell disease (SCD) is the most prevalent severe monogenic disorder worldwide; it is an inherited hemoglobinopathy. This is perhaps why the World Health Organization (WHO) and the United Nations (UN) classified it as a global health challenge. Although the disorder is endemic in tropical areas, it has become a global phenomenon, affecting over 300,000 babies yearly (Chaturvedi *et al.*, 2016; Salinas-Cisneros and Thein, 2020). It is believed that hemolysis is significantly influenced by oxidative stress. Thus, drugs that promote reactive oxygen species (ROS), such as phenylhydrazine (PHZ), can increase lipid peroxidation measured as an elevated malondialdehyde (MDA) level (Ousaaid *et al*., 2022). The spleen's vital tasks include mobilizing or recycling red blood cells and immunological function. Hemolytic anaemia is also associated with an enlarged spleen (splenomegaly); spleen or liver enlargement due to hemolytic anaemia is possible (Innih *et al*., 2020). Phenylhydrazine also stimulates the spleen, decreasing its functions (Kim *et al*., 2012). A non-working spleen may result in a small increase in the number of white blood cells and platelets in the blood, a reduced response to some vaccines and a higher risk of infection.

The first drug to be licensed for use in adults with severe sickle cell anaemia (SCA) was hydroxyurea (HU). After over 30 years, this drug remains the primary treatment option for individuals with SCA. Despite HU being relatively cheap, the drug remains unaffordable in many settings, especially in developing countries. Despite being widely used, hydroxyurea is moderately hazardous, especially over an extended period (Nurain *et al*., 2017; Nardo-Marino *et al*., 2022). The toxic nature of drugs used for maintenance, cost and efficacy necessitated alternative treatment available in medicinal plants. *Moringa oleifera*, a member of the family *Moringacae*, is indigenous to the Indian subcontinent and has since naturalized in many tropical and subtropical

regions of the world. It is a multi-purpose tree because all parts of *Moringa* can be used for different purposes (Kou *et al*., 2018). Antimicrobial, anti-inflammatory, anti-cancer, antidiabetic, antioxidant, hepatoprotective, and cardioprotective capabilities are pronounced disease prevention and therapy associated with *Moringa oleifera* bioactive chemicals (Bhattacharya *et al*., 2018; Wu *et al*., 2021). Only a few reports exist on this medicinal plant's spleen toxicity protection. None of these reports extensively compared the effect of the various parts of *M. oleifera*. This study, therefore, investigated the ability of various parts of *M. oleifera* to modulate PHZ-induced spleen toxicity in male Wistar rats.

Materials and methods

Drugs and chemicals: All chemicals/solutions/reagents utilized were of standard grade.
Plant materials and preparation of Moringa oleifera parts: The Redeemer's University campus in Ede, Osun State, provided fresh *M. oleifera* bark, flowers, leaves, roots, and seeds. Dr. Nodza George, a taxonomist from the Department of Botany at the University of Lagos, verified this before depositing it in the Department's herbarium and giving it a voucher number (ID: LUH 8757). The bark, flower, leaves, roots, and seeds of *M. oleifera* were air-dried before being blended into a powder.
Animals: Male Wistar albino rats weighed between 170 and 220 g and were housed in plastic cages. Rats were housed in 12-hour dark/12-hour light cycles at 25 °C. They received unlimited amounts of water and pelleted rat grower's mash. Prior to treatment, the rats were given a 14-day acclimatization period. The project was granted ethical approval by the College of Medicine, University of Lagos Health Research and Ethics Committee under the title CMUL/ACURE/06/21/863. The rats were handled carefully following the authorized standard protocol of the NIH Guidelines for the Care and Use of Laboratory Animals (NRC, 1985).
Experimental groups: Eight groups of six male Wistar albino rats each were formed from the forty-eight (48) total male rats. Except for the normal control group (NOR), rats were given 40 mg/kg of PHZ intraperitoneally daily for three days to induce anaemia (Sheth *et al*., 2021). Rats with a packed cell volume (PCV) and haemoglobin (HB) less than 36 % and 12 g/dL were deemed anaemic and included in this study. The rats were split into groups I through VIII and they received the following treatment for 28 days:

NOR: Normal rats fed with normal chow
NEG (Negative control): Anaemic rats fed with normal chow
POS (Positive control): Anaemic rats fed with normal chow, plus 25 mg/kg hydroxyurea
MOB: Anaemic rats fed with rat chow blended with 10 % *Moringa oleifera* bark
MOF: Anaemic rats fed with rat chow blended with 10 % *Moringa oleifera* flower
MOL: Anaemic rats fed with rat chow blended with 10 % *Moringa oleifera* leaves
MOR: Anaemic rats fed with rat chow blended with 10 % *Moringa oleifera* root
MOS: Anaemic rats fed with rat chow blended with 10 % *Moringa oleifera* seed

Determination of rats' weight gained: An electronic weighing scale was used to determine the weight of the test rats and this was done weekly. The difference between the mean weights of the rats before and after the experiment was used to calculate weight gained.
Determination of oxidative stress in the spleen: The spleen was removed, cleared of adhering fat and tissue and weighed. A portion of the spleen was homogenized and the concentration of malondialdehyde (MDA) was evaluated spectrophotometrically by estimation of thiobarbituric acid reactive substance (TBARS) as explained by Vashney and Kalle (1990), while nitric oxide (NO) was done according to the method described by Bryan and Grisham (2007). The spleen activity of superoxide dismutase (SOD) was assayed according to Misra and Fridovich (1972) while the activity of catalase (CAT) in the spleen was assayed according to the procedure of Clairborne (1985). The glutathione peroxidase activity was determined following the method described by Rotruck *et al*., 1973. The concentration of reduced glutathione (GSH) in the spleen was assessed by the method of Jollow *et al*., 1974.
Estimation of biochemical disease markers in the spleen: Concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), acid phosphatase (ACP) and albumin were determined using commercially available diagnostic kits (Randox Lab., UK) following manufacturers instruction.
Histopathology: The collected, cleaned, 10 % formalin–fixed, and paraffin–embedded spleen samples were used. Sections were fixed on slides, stained with hematoxylin and eosin, and then examined under a light microscope for interpretation by histopathologists.

Statistical analysis: The experimental data were statistically evaluated using one-way analysis of variance (ANOVA) followed by posthoc Tukey multiple comparison tests (GraphPad Prism software, Inc., San Diego, CA). Values were reported as mean ± SD, and $p < 0.05$ values were deemed statistically significant.

Results

Effect of Moringa oleifera parts on mean body and spleen weight of PHZ-induced anaemic rats: The result of the effect of *Moringa oleifera* parts on the mean body and spleen weight of the experimental rats is shown in Table 1. The percentage of weight gained was lowest in the PHZ-treated group, while the highest was with those fed MOR, 1.81 and 18.13 %, respectively. On the contrary, the PHZ-treated group had an enormous spleen weight of 1.375 ± 0.26 g compared to the normal control group and the HU-treated group with 0.688 ± 0.14 and 0.957 ± 0.28 g, respectively. A significant reduction was noted in the weight of the spleen after treatment with different parts of *M. oleifera*.

Table 1: Effect of *Moringa oleifera* parts on mean body and spleen weight of PHZ-induced anaemic rats

Group	Bodyweight (initial g)	Bodyweight (final g)	%Weight gain (g)	Spleen weight (g)
Normal	190 ± 6.32	212 ± 4.08	11.57	0.688 ± 0.14
Negative	221 ± 2.04^{ac}	225 ± 5.48^{c}	1.81	1.375 ± 0.26^{a}
Hydroxyurea	182 ± 4.08^{ab}	197± 4.08^{ab}	8.24	0.957 ± 0.28^{b}
Bark	210 ± 3.16^{abc}	233± 5.16^{abc}	10.95	0.978 ± 0.05^{b}
Flower	198 ± 2.58^{bc}	228 ± 4.08 ^{abc}	15.15	0.920 ± 0.26^{b}
Leaf	213 ± 5.16^{abc}	238 ± 4.08 ^{abc}	11.74	1.137 ± 0.13^{a}
Root	193 ± 2.58^{bc}	228 ± 4.08 ^{abc}	18.13	0.920 ± 0.09^{b}
Seed	172 ± 2.58^{abc}	203 ± 6.06^{c}	18.02	0.930 ± 0.12^{b}

Key *a: significant when compared to control, b: when compared to anaemic control, and c: when compared to hydroxyurea (P < 0.05) indicates significant change by Tukey multiple range ANOVA.*

Effect of Moringa oleifera parts on oxidative stress markers and antioxidant status: The effect of *M. oleifera* on the antioxidant values of the PHZ-induced anaemic rats was presented in Figure 1. SOD, CAT, GPx and GSH were used to monitor oxidative stress in the spleen. No significant difference was seen between the SOD values of the normal rats (0.12 ± 0.01) and the anaemic rats (0.11 ± 0.00). However, the flower and root treatment groups showed significantly higher SOD levels than the HU treatment group. HU treatment brought the CAT values to those of normal control, while the *M. oleifera* treatments also elevated the decline experienced in anaemic rats but were higher than in the HU rats. However, there was a significant ($p < 0.05$) increase in SOD, CAT levels, decrease in GPx in all the groups treated with the plant materials in comparison with the negative control.

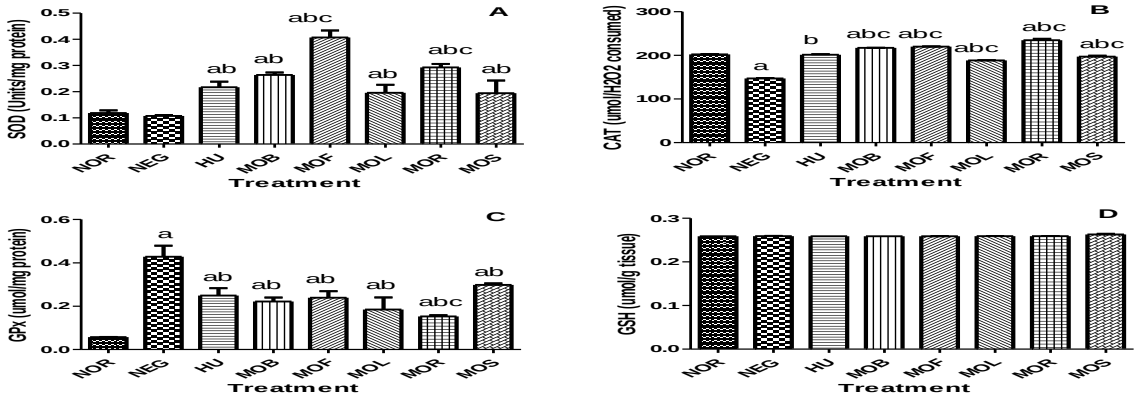

Figure 1: *Effect of Moringa oleifera parts on (A) Superoxide dismutase (SOD), (B) Catalase (CAT), (C) Glutathione peroxidase (GPx) and (D) GSH of rats' spleen. NOR: Normal control; NEG: anaemic group; HU: hydroxyurea (standard drug); MOB: Moringa oleifera bark-fed rat; MOF: Moringa oleifera flower-fed rat; MOL: Moringa oleifera leaf fed rat; MOR: Moringa oleifera root fed rat; MOS: Moringa oleifera seed fed rat. a: significant when compared to control, b: when compared to anaemic control, and c: when compared to hydroxyurea.*

Nitric oxide (NO), lipid peroxidation (MDA), total protein and albumin were significantly ($p < 0.05$) elevated in negative control relative to the normal control (Figure 2). For the NO, the administration of hydroxyurea (25 mg/kg) and *Moringa oleifera* parts significantly ($p < 0.05$) attenuated NO levels compared to the negative

control rats. The only exception is with seeds that showed elevated levels higher than both the normal control rats and HU-treated rats. The elevated MDA in the anaemic control (10.81 ± 0.60) was reduced with HU (8.25 ± 0.53) and other treatments but not enough to be as the normal control value (4.66 ± 0.14). Only MOL treatment (4.59 ± 0.11) could bring the values to that of control rats. There appear to be no changes in the total protein in all the groups but the elevated albumin level in the anaemic rats was reduced by HU, MOB, MOL, MOR and MOS while MOF reduced it further to that of control rats.

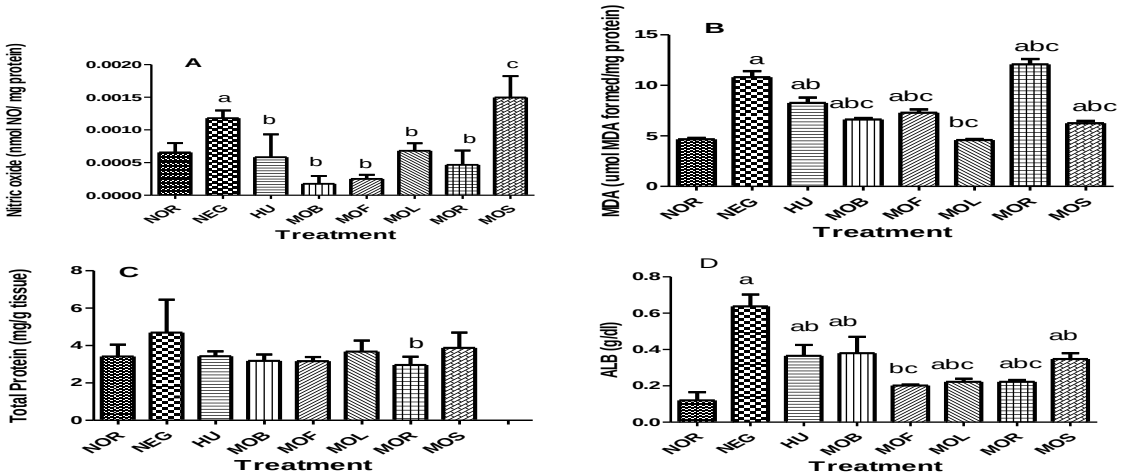

Figure 2: *Effect of Moringa oleifera parts on nitric oxide, lipid peroxidation (MDA), total protein and albumin (ALB) of rats' spleen. NOR: Normal control; NEG: anaemic group; HU: hydroxyurea (standard drug); MOB: Moringa oleifera bark-fed rat; MOF: Moringa oleifera flower-fed rat; MOL: Moringa oleifera leaf fed rat; MOR: Moringa oleifera root fed rat; MOS: Moringa oleifera seed fed rat. a: significant when compared to control, b: when compared to anaemic control, and c: when compared to hydroxyurea.*

A significant elevation was observed in ALT, ALP and ACP levels in the anaemic rats compared to the control (Figure 3). In the ALT assay, HU and MOB treatments lowered the elevated value in anaemic rats to that of control rats while other treatments could not. All the treatments reduced the elevated ALP value recorded in the NEG group (anaemic rats), but none could place it at the control rat's value. For the acid phosphate (ACP), MOB (24.69 ± 0.59) and MOS (31.18 ± 0.80) lowered the elevated level in NEG (98.76 ± 0.68).

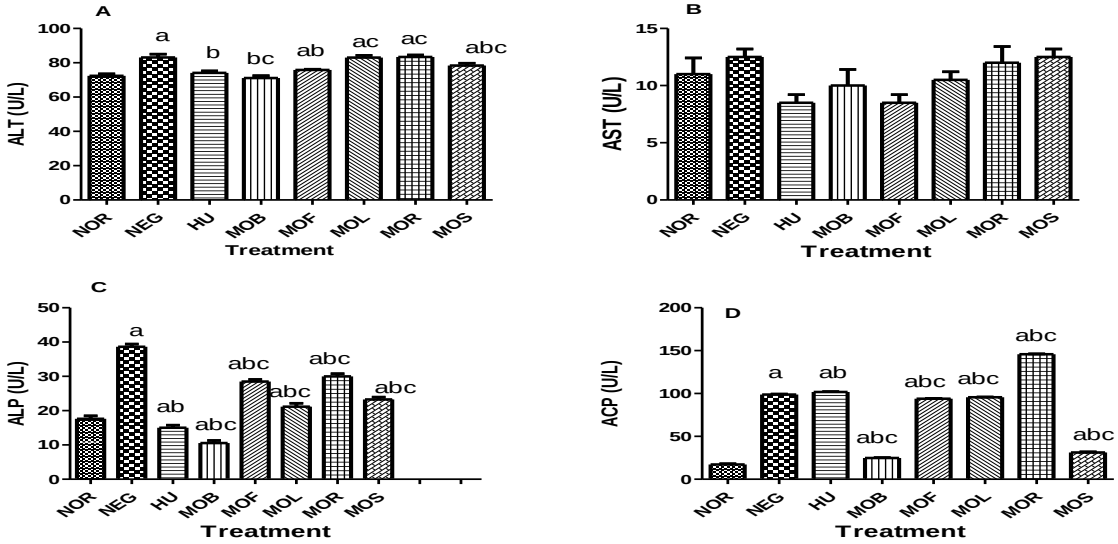

Figure 3: *Effect of Moringa oleifera parts on (A) ALT, (B) AST, (C) ALP and (D) ACP of rats' spleen. ACP: acid phosphatase; ALP: alkaline Phosphatase; ALT: alanine aminotransferase; AST: aminotransferase; CAT: catalase; NOR: Normal control; NEG: anaemic group; HU: hydroxyurea (standard drug); MOB: Moringa oleifera bark-fed rat; MOF: Moringa oleifera flower-fed rat; MOL: Moringa oleifera leaf-fed rat; MOR: Moringa oleifera root fed rat; MOS: Moringa oleifera seed-fed rat, a significant when compared to the control; b when compared to anaemic control; and c when compared to the hydroxyurea.*

Histopathology result: The histology sections of the spleen are shown in Figure 4. The results indicate normal spleen architecture in the normal control rats with red pulp, white pulp and a splenic artery (Figure 4A). In the anaemic rats (Figure 4B), the photomicrograph indicates a haemorrhage area and an aggregate of inflammatory cells. However, in the groups treated with *Moringa oleifera* parts (Figures 4 D (MOB), E (MOF), F (MOL), G (MOR) and H (MOR)), the result showed moderate/mild focal area of haemorrhage change indicating improvement in the spleen.

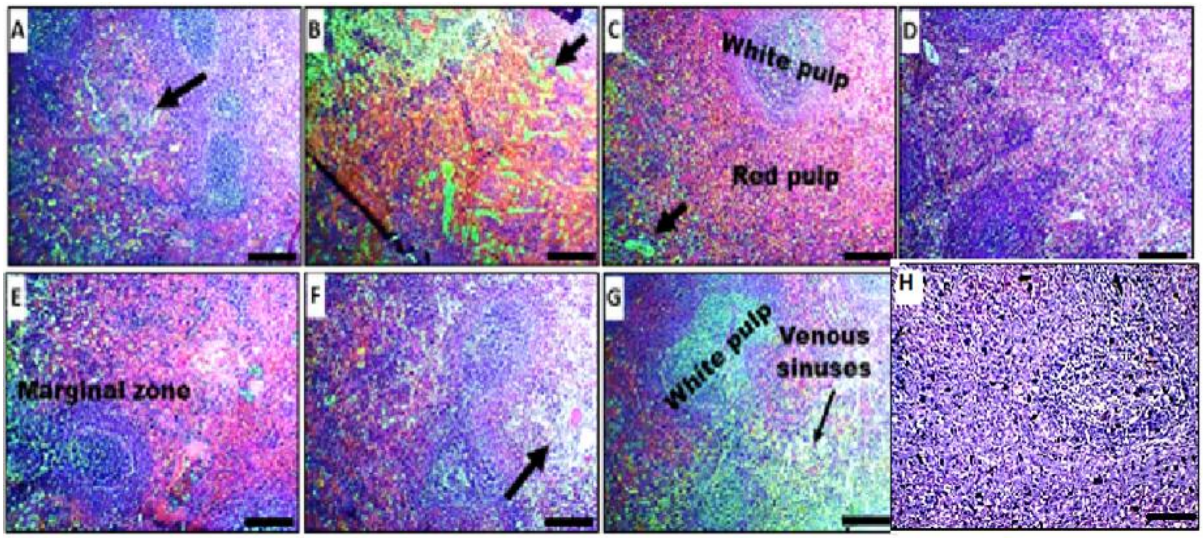

Figure 4: *Photomicrographs of the panoramic views of spleen general micromorphological presentations in adult Wistar rats across the study groups: Hematoxylin and Eosin stain (Scale bar 25 µm). Big black arrowheads indicate altered micromorphology, fibrosis, degenerating cells of red and white pulps across affected groups, loss of connective tissue, infiltrated splenic stroma and parenchyma. (Magnification x 400).*

Discussion

According to Cui *et al.* (2018), one of the primary effects of increased ROS production in physiological systems is the formation of malondialdehyde (MDA). Therefore, MDA is a sign of increased lipid oxidation due to oxidative stress in the body resulting from the excessive production of free radicals in the form of ROS. In the current investigation, lipid peroxidation (MDA), nitric oxide, total protein and albumin levels were higher in the spleens of the PHZ-induced rats. However, they were much lower in the rats fed with *Moringa oleifera* parts (MOB, MOF and MOL) than in the negative control, likely due to the plant's high antioxidant potential. Emuejevoke *et al.* (2016), who worked on *Moringa oleifera* leaf extract, reported similar results; dry beet green or beetroot pomace biscuit also reduced the level of lipid peroxidation, nitric oxide total protein and albumin in anaemic rats induced by PHZ (Elaby and Ali, 2018; Abdo *et al*., 2021). Part of the internal defense system in animals is the enzymatic antioxidant system, which includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Generally, suppressing this system leads to pro-oxidant status and consequent damage to the cells, organs or tissues, as applicable (Galasso *et al*., 2021). As a result, superoxide radicals and hydrogen peroxide may accumulate in the spleen of rats exposed to PHZ due to the decrease in SOD and CAT activities (Moriasi *et al*., 2020; Aloke *et al*., 2021). The restoration of SOD and CAT values by *Moringa oleifera* leaves, seed and bark demonstrated that the plant materials have tremendous antioxidant potential than hydroxyurea.

Additionally, by raising the amount of glutathione peroxidase (GPx) above normal, PHZ increased oxidative stress in the spleen. However, treatments with HU and *Moringa oleifera* components alleviated the organ's oxidative damage with the root, leaves and bark performing better than hydroxyurea. As an alternative to catalase, the detoxification of hydrogen peroxide to oxygen and water can be catalyzed by glutathione peroxidase (Ighodaro and Akinloye, 2018). In this study, all these enzymes were modulated effectively in the spleen by various parts of the *Moringa oleifera*, particularly the leaves and the bark. The spleen plays a role in immunological activity, red blood cell mobilization and recycling (Liang *et al*., 2023). It was hypothesized that any substance affecting the histology of the spleen would adversely affect red blood production. Some

substances or medications, including PHZ, have been reported to reduce spleen function (Sheth *et al*., 2021), which is similar to asplenia and hyposplenism, sometimes known as a non-functional spleen and a partially functioning spleen associated with congenital disabilities, trauma injuries and sickle cell anaemia (Peretz *et al*., 2022). These medical conditions may result in a slight increase in blood counts of white blood cells and platelets a decreased response to some vaccines and an increase in infection susceptibility (Jia and Pamer, 2009). The control rats in this study had normal red pulp, white pulp and spleen arteries, according to histological sections of the spleen tissues which is consistent with the findings of Sheth *et al*. (2021). The several degenerations, severe hemorrhagic area and moderate aggregation of inflammatory cells seen in PHZ-treated rats might be a sign of the harmful effects of the treatment, thus changing the functional efficiency of the spleen. Conclusively, a modest focal region of haemorrhage, a mild area of fatty change and an absence of inflammation evidenced by *M. oleifera* treated rats suggested lessening the spleen damage by the plant materials.

Conclusion

This present study reported that *Moringa oleifera's* bark, flower, leaves, root and seed have significant anaemic-fighting potential against PHZ-induced haemolytic anaemia, supporting their folklore use in alternative medicine. The flower and bark demonstrated the best activity against weight loss and spleen enlargement associated with anaemic conditions. The leaf, seed and bark prevented lipid peroxidation, while only the duo of leaf and bark modulated antioxidant enzymes effectively in the organ examined. The histopathology results also supported the biochemical results, as *Moringa oleifera* bark, leaf, root, flower, and seed reduced the PHZ toxicity's effects on the rats' spleen. However, the leaf is the most potent and effective part based on the results of these findings.

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