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Antioxidative and Antianaemic Potentials of Aqueous Leaf Extract of Lowveld Bead String *(Alchornea laxiflora)* in Phenylhydrazine-induced Anaemic Wistar Rats and Its Phytochemical Constituents

Awolusi, Oluwasola Michael[1], Ojelabi, Adetutu Omolola[1], Otenaike, Oluwakemi[1], Ibrahim, Omeyiza Micheal[2], Ayotunde, Bolatito Ruth[1], Awonubi, Christopher Opeoluwa[3], Bamisaye, Fisayo Abraham[1]

[1]Department of Biochemistry, Faculty of Science, Federal University Oye-Ekiti, Oye-Ekiti, Ekiti State, Nigeria.
[2]Department of Biochemistry, Faculty of Science, Confluence University of Science and Technology, Osara, Kogi State, Nigeria.
[3]Department of Chemical Science, Faculty of Science, Yaba College of Technology, Yaba, Lagos, Nigeria

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*Corresponding Author:
Awolusi, Oluwasola Michael
Email: awolusioluwasola@gmail.com
Tel:+234809185040

ABSTRACT

Background: Anaemia, a public health concern, is characterized by a decrease in red blood cell count, hemoglobin, and packed cell volume. Phenylhydrazine, a toxic chemical, is commonly used to induce anaemia in experimental models. This study investigated the antioxidative and antianaemic effects of aqueous leaf extract of *Alchornea laxiflora* in phenylhydrazine-induced anaemic wistar rats.

Method: Qualitative phytochemical screening and HPLC analysis of the extract were carried out. Thirty female wistar rats weighing between 166-169 g were randomly divided into six (6) groups of five animals each. Groups 1 and 2 served as negative and positive control respectively and treated with 1 mL/kg body weight of distilled water only. Groups 2 to 6 were induced with anaemia by intraperitoneal injection of 36 µL/kg body weight of phenylhydrazine (PHZ). Groups 3 to 5 were treated with 500, 1000 and 1500 mg/kg body weight of aqueous leaf extract of *A. laxiflora* respectively while group 6 was treated with 2 mg/kg body weight of folic acid. The experiment lasted for 14 days after which the rats were sacrificed and their blood collected for hematological parameters. Antioxidative effects of the extract was also investigated.

Results: The results showed that the aqueous leaf extract of *Alchornea laxiflora* is rich in phytochemicals. The extract demonstrated antioxidative properties, as evidenced in its reductive effects on oxidative stress. It also revealed antianaemic effect where it significantly improved hematological parameters such as packed cell volume (PCV), haemoglobin (HB), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), and red blood cell (RBC).

Conclusion: This study confirmed the presence of various phytochemicals in the aqueous leaf extract of *Alchornea laxiflora.* The extract demonstrated antioxidative and antianaemic properties. The extract's protective effects against anaemia are likely attributed to its phytochemical constituents.

Introduction

Nature has tremendously blessed its occupants with medicinal plants that can cure or manage one disease or the other. This may be due to their phytochemicals that are believed to have antioxidative potentials. Oxidation reactions in living cells can lead to series of ailments[1].

Anaemia, occurs when the body has fewer red blood cells than normal[2,3]. This may be as a result of haemoglobin defects that impede haemoglobin's function, a decrease in the quantity of haemoglobin available for oxygen transport, or a lower-than-normal number of red blood cells[4]. The symptoms of slow-onset anaemia are often subtle, manifesting as chronic fatigue, muscle weakness, headaches, and reduced stamina. The size and quantity of haemoglobin in each red blood cell can also be used to categorized anaemia[5]. The most prevalent blood condition, anaemia, affects between 5 and 30% of people worldwide[6]. The World Health Organisation (WHO) estimated that around 40 % of children aged 6-59 months, 37 % of pregnant women, and 30 % of women 15-49 years old are affected. In 2019, 30 % (539 million) of non-pregnant women and 37 % (32 million) of pregnant women aged 15-49 years were anaemic. This condition predominantly affects children, but it also disproportionately impacts the elderly and women of reproductive age, particularly in pregnancy. One of the six World Health Organization (WHO) global nutrition targets for 2025 is anaemia[6].

Conventional anaemia treatments exist, but they often come with unwanted side effects, hence the search for alternatives that have little or no side effects. In this regard, traditional users of Lowveld bead string (*Alchornea laxiflora*) believed in its antioxidative and antianaemic effects.

Alchornea laxiflora belongs to the Euphorbiaceae family, also called spurges., includes monoecious and dioecious plants, shrubs, vines, and trees. Major groupings in this family are distributed globally and contain latex[7]. *A. laxiflora* is widely spread in evergreen forests and grow from sea level to 1600 m above sea level[8].

Developing countries often rely on medicinal plants for treating anaemia due to a combination of economic, cultural, and healthcare system factors, which include; cost and accessibility, traditional knowledge, health care infrastructure as they have limited access to health facilities, perceived safety and natural appeal. This study explores the scientific basis of the antioxidative and antianaemic potentials of aqueous leaf extract of *Alchornea laxiflora* in anaemic wistar rats.

Justification

The orthodox drugs that are in use for the treatment of anaemia are not without side effects, hence the search for alternatives that are of little or no side effects. Traditional users of *Alchornea laxiflora* believed in its antianaemic effect. This research will therefore look into the scientific reason behind the use of *Alchornea laxiflora* locally for the treatment of anaemia. Furthermore, this research will investigate the efficacy of aqueous leaf extract of the plant as an anti-anaemic agent.

Materials and methods

Materials

Reagents and chemicals: Phenylhydrazine was obtained from Central Drug House (CDH) Ltd Corp, office 7/28 Vardaan House Daryaganj New Delhi. All other reagents were of analytical grade.

Plant material: *Alchornea laxiflora* leaves were collected from a local farm in Oye Ekiti, Ekiti State, Nigeria at about 9am, in the month of April. It was authenticated in the herbarium Unit department of the Department of Plant Science and Biotechnology, Ekiti State University, Ado Ekiti, Nigeria with the voucher specimen number: UHAE 2023044.

Experimental animals: Thirty female wistar rats (166-169g) were obtained from Animal House of the Federal University Oye Ekiti's animal house. They were housed in clean cages, acclimatized for 2 weeks, and fed *ad libitum* with standard diet and distilled water.

Methods

Preparation of aqueous leaf extract of *Alchornea laxiflora.* The plant leaves were dried at room temperature until constant weight was obtained, grounded into powder, and 1 kg was extracted with 10 L of distilled water for 24 hours. The mixture was filtered, freeze-dried, and the obtained powder stored in a glass container for further analysis.

Qualitative phytochemical screening of aqueous leaf extract of *Alchornea laxiflora*: Each phytochemical was screened as follow:

Terpenoids: Salkowski test was used, where 5 ml of extract was mixed with 2 ml of chloroform, and concentrated sulphuric acid was carefully added to form a layer. Formation of a reddish brown colouration on the interface

indicates the presence of terpernoid[9].

Phenols: Ferric Chloride test was employed. Extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols[10].

Saponin: Foaming test was used, where 0.5 g of extract was shaken with 5 ml of distilled water and then heated to boil. If foam produced persists for ten minutes it indicates the presence of saponins[11].

Steroids: Few drops of acetic anhydride were added to the sample, then, few drops of concentrated sulfuric acid was added, a reddish-brown colour indicate the presence of steroids[12].

Flavonoids: Dissolved in diluted NaOH and HCL was 0.2 gm of extract. A yellow solution that turns colourless indicates presence of Flavonoids[10].

Glycosides: Ten (10) ml of 50% H_2SO_4 was added to 1 g of the extract in a tube. The mixture was heated in boiling water for 15 minutes, 10 ml of fehlings solution was added and the mixture was boiled. A brick-red precipitate observed in each extract tested indicated the presence of glycosides in the extract[13].

Tannins: Small quantity of extract was mixed with water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green solution indicated the presence of tannins [14].

Phlobatannins: Few drops of ferric chloride solution were added to the sample, blue, green, or purple colour indicated the presence of phlobatannins [15].

Anthraquinones: 1 mL of 5 % potassium hydroxide solution was added to the sample in a test tube and heated gently, pink colouration indicated the presence of anthraquinones[16].

Structural elucidation of aqueous leaf extract of *Alchornea laxiflora* using HPLC: The structural elucidation of aqueous leaf extract of *A. laxiflora* was carried out using HPLC[17].

Induction of anaemia: Haemolytic anaemia was induced by intraperitoneal (I.P.) injection of 36 µL/Kg body weight of phenyl hydrazine (PHZ) once for 2 days. This was confirmed after taking the rats' blood samples for PCV test[18].

Experimental design: The experimental rats (30) weighing between 166-169 g were randomly divided into six (6) groups of five animals each. The treatment lasted for 14 days. The design was as shown below:

Group 1: (Negative control): Non-anaemic, treated with 1 mL/kg of distilled water only.

Group 2: (Positive control): Anaemic rats, treated with 1 mL/kg of distilled water only.

Group 3: Anaemic rats, treated with 1 mL of 500 mg/kg body weight of aqueous leaf extract of *A. laxiflora*.

Group 4: Anaemic rats, treated with 1 mL of 1000 mg/kg body weight of aqueous leaf extract of *A. laxiflora*.

Group 5: Anaemic rats, treated with 1 mL of 1500 mg/kg body weight of aqueous leaf extract of *A. laxiflora*.

Group 6: Anaemic rats, treated with 1 mL of 2 mg/kg body weight of standard drug (Folic acid).

Determination of superoxide dismutase activity: The enzyme Superoxide dismutase has the ability to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA in the pH 8.2 is 50%. The principle of this method is based on the competition between the pyrogallol autoxidation by O_2^- and the dismutation of this radical by SOD[19].

Determination of catalase activity: Catalase is able to decompose hydrogen peroxide by two different reaction pathways. In the first, known as the "catalatic" pathway, 2 molecules of hydrogen peroxide are converted to water and oxygen (catalatic activity)[20].

Determination of malondialdehyde concentration: This assay was based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25° C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm[21].

Determination of glutathione concentration: These spectrophotometric procedures were based on the method of[22], who reported that 5,5'-dithiobis- (2,-nitrobenzoic acid) is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH.

Blood collection: The method of[23] was employed.

Determination of haematological parameters: These tests were carried out according to[24] Hoffmann *et al.,* (2018) using automated haematology analyser.

Statistical Analysis

All analyses were carried out in 5 replicates and data were subjected to Analysis of Variance (ANOVA) using Graph Pad prism. Statistical differences between mean values were determined by Tukey's post hoc test and accepted at $P < 0.05$. Values were expressed as mean ± S.E.M.

Results

The phytochemical analysis of *A. laxiflora* leaf extract showed the presence of cardiac glycosides, saponins, tannins, flavonoids, steroid, terpeniod and phenols. Anthraquinone and phlobatannin were not detected in this extract (Table 1).

Table 1: Phytochemical screening of aqueous leaf extract of *A. laxiflora*.

Phytochemicals	Status
Saponins	+
Tannins	+
Phlobatannins	-
Anthraquinones	-
Steroids	+
Terpenoids	+
Flavonoids	+
Glycosides	+
Phenols	+

Note: Positive sign (+) represents presence while negative sign (–) represents absence of the phytochemicals.

HPLC analysis revealed the presence of various phytochemicals, with concentrations ranging from 0.141 to 35.641 mg/g. The major constituents included phyto (35.641 mg/g), justicioside A (4.599 mg/g), and kaempferol (1.54 mg/g). Other compounds present in lower concentrations were gamma tocopherol, justicinol, jubetolin, allantoin, Vitexin (0.141 mg/g) and others (Table 2).

Table 2: HPLC characterization of aqueous leaf extract of *A. laxiflora*

Components	Retention Time	Peak Area	Peak Height	Concentration (mg/g)
Terpenoids				
Phytol	3.700	2621.314	52.516	35.641
Alpha-Amyrin	10.500	133.455	5.742	0.191
Beta-Amyrin	9.116	117.135	5.742	0.168
Steroids				
Campesterol	10.500	201.420	5.531	0.288
Gamma Tocopherol	5.883	767.619	13.163	1.099
Flavonoids				
Vitexin	12.816	101.315	5.559	0.141
Kaempferol	15.500	1078.000	16.944	1.543
Apigenin	20.500	197.376	6.236	0.283
Glycosides				
Justicioside A	17.233	3212.620	40.018	4.599
Vitexin	12.816	101.315	5.559	0.141
Phenols				
Umbeliferone	13.466	109.393	5.279	0.157

Alkaloids				
Quinindoline	17.233	122.886	5.370	0.176
Vasicinone	11.850	180.490	5.643	0.258
Vasicinol	21.416	191.053	5.608	0.151
Jusbetolin	19.400	234.267	8.369	0.466
Justicinol	15.500	722.480	13.466	1.034
Allantoin				
Allantoin	11.850	103.704	5.643	0.258

Figures 1a-d reveal the antioxidative effects of aqueous leaf extract of *A. laxiflora* in the serum of phenylhydrazine-induced wistar rats.

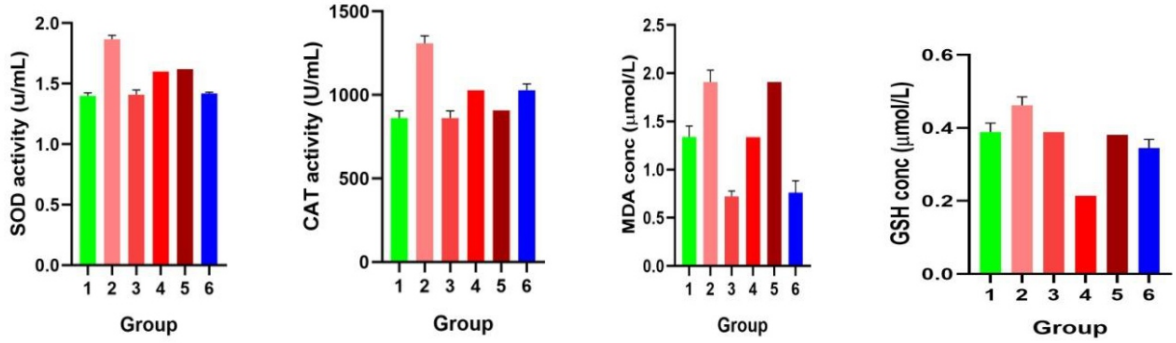
No significant difference in serum SOD activity was observed in rats treated with 500 mg/kg while its significant ($P < 0.05$) increases were noted in rats treated with 1000 and 1500 mg/kg body weight of the extract, compared with the negative control (Fig 1a).

CAT activity in the serum of rats treated with 500 mg/kg remained unchanged but significantly ($P < 0.05$) increased in rats treated with 1000 and 1500 mg/kg body weight of the extract, and 2 mg/kg body weight of the folic acid compared with the negative control (Fig 1b).

A significant ($P < 0.05$) decrease in MDA level was observed in rats treated with 500 mg/kg body weight of the aqueous leaf extract and 2 mg/kg body weight of the folic acid, compared with the negative control. However, MDA level remained unchanged in rats treated with 1000 mg/kg body weight of the extract, but significantly ($P < 0.05$) increased in those treated with 1500 mg/kg body weight of the extract (Fig 1c).

A significant ($P < 0.05$) decrease in GSH level was observed in rats treated with 1000 mg/kg body weight of the aqueous leaf extract, compared with the negative control. In contrast, GSH level remained unchanged in rats treated with 500 and 1500 mg/kg body weight of the extract, and 2 mg/kg body weight of the folic acid (Fig 1d).

However, the activities of SOD and CAT, and the concentrations of MDA and GSH significantly ($P < 0.05$) increased in the positive control rats when compared with the negative control and the treated rats (Fig 1a-d).



1. Negative control
2. Positive control
3. 500 mg/kg extract
4. 1000 mg/kg extract
5. 1500 mg/kg extract
6. 2 mg/kg extract

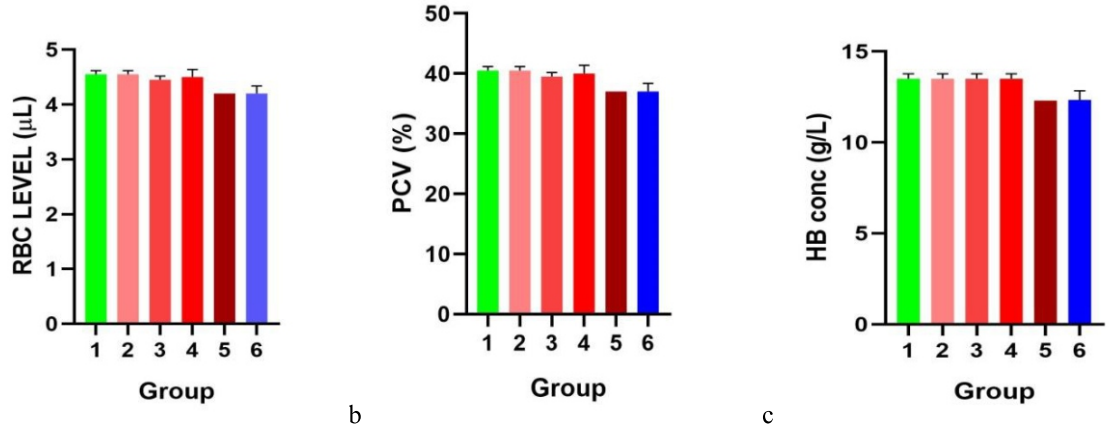
Figure 1: Effects of aqueous leaf extract of *A. laxiflora* on the activities of (**a**) SOD and (**b**) CAT, and concentrations of (**c**) MDA and (**d**) GSH in the serum of phenylhydrazine-induced anaemic wistar rats.

Figures 2a-g reveal the haematological effects of aqueous leaf extract of *A.laxiflora* in the serum of phenylhydrazine-induced wistar rats.

No significant differences were observed in red blood cell (RBC) levels between rats treated with 500 and 1000 mg/kg body weight of the aqueous leaf extract, compared with the negative and positive controls. However, a significant ($P < 0.05$) decrease in RBC levels was noted in rats treated with 1500 mg/kg body weight of the extract and 2 mg/kg body weight of the folic acid, relative to the negative and positive controls (Fig 2a).

Packed cell volume (PCV) levels showed no significant differences after treatment with 500 and 1000 mg/kg body weight of the aqueous leaf extract compared with the negative and positive controls. In contrast, a significant ($P < 0.05$) decrease in PCV level was observed in anaemic rats treated with 1500 mg/kg body weight of the extract and 2 mg/kg body weight of the folic acid, relative to the negative and positive control (Fig 2b).

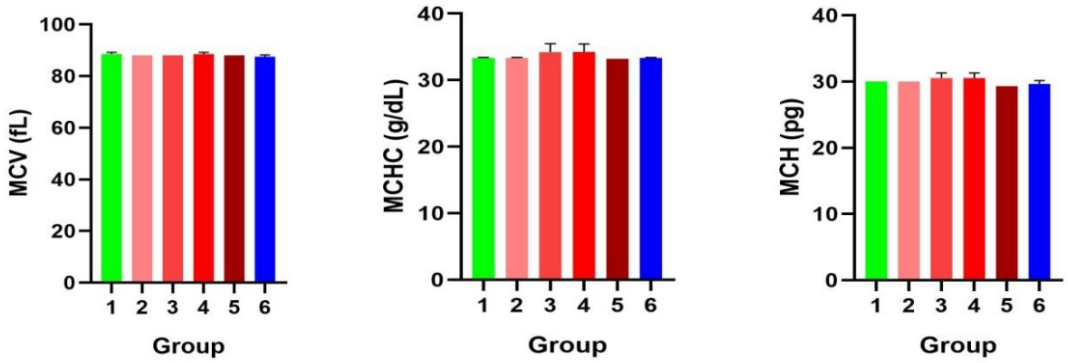
No significant differences were observed in hemoglobin (HB) levels in the blood of rats treated with 500 and 1000 mg/kg body weight of the aqueous leaf extract compared with the negative and positive controls. However, a significant ($P < 0.05$) decrease in HB level was noted in rats treated with 1500 mg/kg body weight of the extract and 2 mg/kg body weight of the folic acid, relative to the negative and positive controls (Figure 2c).



a b c

1. Negative control
2. Positive control
3. 500 mg/kg extract
4. 1000 mg/kg extract
5. 1500 mg/kg extract
6. 2 mg/kg extract

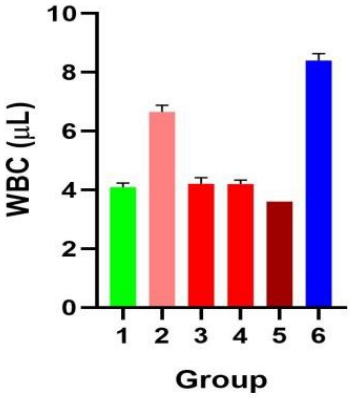
No significant differences were observed in MCV, MCH, and MCHC levels among the treated anaemic rats compared with the positive control (Fig 2d-f).



d e f

1. Negative control
2. Positive control
3. 500 mg/kg extract
4. 1000 mg/kg extract
5. 1500 mg/kg extract
6. 2 mg/kg extract

Treatment with 1500 mg/kg body weight of the aqueous leaf extract of *A. laxiflora* significantly ($P < 0.05$) decreased WBC levels. In contrast, 500 and 1000 mg/kg body weight of the extract treatments showed no significant differences in WBC levels compared with the negative control. Additionally, rats treated with 2 mg/kg body weight of the folic acid significantly ($P < 0.05$) possessed an increased WBC level compared to the negative control. However, WBC levels significantly ($P < 0.05$) increased in the positive control when compared with the negative control and the rats treated with leaf extract (Fig 2g).



g

1. Negative control
2. Positive control
3. 500 mg/kg extract
4. 1000 mg/kg extract
5. 1500 mg/kg extract
6. 2 mg/kg extract

Figure 2: Effect of aqueous leaf extract of *A. laxiflora* on (**a**) RBC, (**b**) PCV, (**c**) HB, (**d**) MCV, (**e**) MCHC, (**f**) MCH and (**g**) WBC of phenylhydarzine-induced anaemic wistar rats.

Discussion

Herbal remedies have been utilised for ages and are thought to contain bioactive compounds. Local herbalists are also said to have used crude extract from herbs and medicinal plants to cure a variety of illnesses[25]. Secondary metabolites such as steroids, phenolics, alkaloids, flavonoids, present in the aqueous leaf extract of *Alchornea laxiflora*, have been shown to have antioxidative, antianaemic and antimicrobial properties[26]. Flavonoids, phenolics, and tannins, are well-known for their antioxidant qualities[27]. These substances scavenge reactive oxygen species (ROS) and free radicals, which can harm blood cells through oxidative stress[28]. The antioxidant effects of this extract are likely due to its phytochemical constituents, including polyphenols, alkaloids, and flavonoids. The work of[29] proved this discovery as the plant extract scavenged free radicals.

The observed increased activities of CAT and SOD in rats treated with 1000 and 1500 mg/kg body weight of the extract may be may be due to the fact that the antioxidant activity of this extract and its mitigation of the harmful effects of reactive oxygen species (ROS) is concentration dependent, as earlier proven by[30]. A compound like the phytol, present in the extract, as observed in its HPLC analysis, may directly or indirectly stimulate the expression of SOD activity through different biochemical pathways[31].

The aqueous leaf extract of *Alchornea laxiflora* appeared to have facilitated the restoration of normal erythropoiesis and the reduction of oxidative stress linked to anaemia by easing the anaemia induced by phenylhydrazine as shown in haematological analysis of this study. As part of the healing process, this improvement caused a compensatory rise in SOD levels, shown in antioxidative effects of the extract which has also been proved by the work of[32].

In **hemolytic anaemia**, where red blood cells (RBCs) are destroyed prematurely, oxidative stress performs a major role. Breakdown of RBCs can increase production of free radicals, leading to lipid peroxidation in the cell membranes. This results in the formation of MDA, which can further damage RBCs and other cellular components, exacerbating the condition[33]. This study showed that the aqueous leaf extract of *Alchornea laxiflora* may have strong antioxidant qualities due to the observed lowered MDA level in the extract-treated rats which may have been achieved by preventing lipid peroxidation. This observation corroborated by[33].

A non-immunogenic chemical called phenylhydrazine selectively destroys mature red blood cells through oxidative stress, thereby denaturing red cell haemoglobin, membrane phospholipids, and energy metabolism-related enzymes. This effect is produced in the presence of oxygen and results to hemolytic type of anaemia[34]. The significant increase in hematological indices (PCV, RBC, MCH, MCHC) in the extract-treated rats supports the traditional use of *A. laxiflora* in treating anaemia. This extract may have exerted anti-anaemic effects due to the presence of polyphenols, alkaloids, and flavonoids present in it. These phytochemicals primarily increased iron absorption, decreased iron excretion, and promote the accumulation of excess iron in tissues, which aid the steady production of red blood cells[35].

The observed increased white blood cell count in response to the administration of this extract in rats may have been brought about by the oxidative damage caused to red blood cells through the action of phenylhydrazine, which may have provoked a compensatory response in the rats' body which led to the increased white blood cells in the rats. This result was similar to the work of[36]. Additionally, phenylhydrazine may have initiated haemolysis and triggered the immune system. This may have led to an observed increase in white blood cell production in the body of these anaemic rats. This result is also similar to that of[37]. This may have helped fight off the perceived threat and aided in the elimination of debris and injured cells in the extract-treated rats. This was also similar to the work of[38].

Conclusion

This study confirmed the presence of various phytochemicals in the aqueous leaf extract of *Alchornea laxiflora* as revealed by HPLC analysis. The extract demonstrated antioxidative and antianaemic properties. The protective effects of aqueous leaf extract of *A. laxiflora* against oxidation and anaemia are likely attributed to its phytochemical constituents.

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