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Anti-anaemic effects of leaf protein concentrate of *Eremomastax speciosa* (Hochst) Cufod. on some blood parameters of male Sprague-Dawley rats exposed to phenylhydrazine-induced anaemia

§, 1, 4 **Nwaigwe Chukwuemeka Onyekachi**, ² **Nwaigwe Chioma Uchenna**, ³ **Obiekwe Uchechukwu Chukwudi**, ⁴ **Raji Lukman Oladimeji**

¹Department of Animal Health and Production, Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

²Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

³Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

⁴Department of Theriogenology and Production, Faculty of Veterinary Medicine, University of Ilorin, Kwara State

§**Corresponding author:** Nwaigwe Chukwuemeka Onyekachi; E-mail/Phone:
chukwuemeka.nwagwe@unn.edu.ng /+234 8054871461

Abstract

This study investigated the effects of leaf protein concentrate (LPC) of *Eremomastax speciosa* (Hochst.) Cufod on some haematological and serum profile of 45 male Sprague-Dawley rats divided into nine groups of five rats per group. The rats received fresh drinking water daily, with feed at 85% of ad-lib. The standard haematinic used was Ranferon-12®, at the dose of 0.3ml/kg body weight. The resultant groups were; Group1 (Positive control, received commercial feed) Group 2 (phenylhydrazine only or negative control), Group 3 (anaemia +haematinic), 4, 5 and 6 (anaemia +100 mg/kg, 200 mg/kg and 400 mg/kg of LPC respectively), Groups 7, 8, 9 (Untreated (or UT) +100 mg/kg, 200 mg/kg and 400 mg/kg of LPC respectively). The LPC was administered orally to the designated groups, while the controls received distilled water in place of the LPC. Anaemia was induced by intraperitoneal injection of phenylhydrazine at 40 mg/kg for 2 days. Samples for haematological and biochemical analysis were collected on days 9, 21 and 28 of the experiment. Results showed that the groups supplemented with LPC were better in terms of the haematology and serum biochemical values compared to the negative control, and this strongly supported the traditional use of *E. speciosa* leaf in the treatment of anaemia.

Keywords: *Eremomastax speciosa*, Anaemia, Haematinic, Phenylhydrazine, Haematology.

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INTRODUCTION

Eremomastax speciosa (Hochst.) Cufod. is a tropical, stout and erect multi-branched herb, used widely on a folkloric basis in the tropical regions of Nigeria and Cameroon, where they are mostly found (Hotez, and Molneux, 2008); Ndem *et al.*, 2013). The plant is edible, belongs to the Acanthaceae family, and are grown in the farmyards of most rural communities for medicinal and ornamental purposes. Among the traditional Ibibios in the Cross River state, the plant is commonly known as Edem iduduot, whereas among the Akwa Ibom and Efiks, it is called Ikpo ikong (Ndem *et al.*, 2013). According to the World Health Organization (WHO), more than 80% of the people in developing countries are relying on the traditional remedies of plant origin for their health care (Velavan, 2015) hence, Phytomedicines have formed a viable link between the traditional and modern medicine (Balamurugan, *et al.*, 2019). The plant is used by traditionalists in combination with other herbs, to treat a host of diseases such as fertility cases in women (Essien *et al.*, 2020), dysentery and urinary tract infections diarrhoea, and anaemia (Oben *et al.*, 2006; Essien *et al.*, 2020). However, numerous information concerning medicinal plants is generally passed through oral tradition by the herbalists, which are devoid of physically documented information that should serve as a guide for the use of herbal remedies (Salako *et al.*, 1990). Leaf protein concentrate (LPC) are made by concentrating the nutrients in the leaf of a particular plant, which can then be effectively used as a supplement, and this product have been reported to have huge advantage in human animal nutrition, and also in combating malnutrition-related diseases such as hypoproteinaemia and anaemia in humans, (Lowe, 2002; (Nwokoro *et al.*, 2022). Leaves of any edible plant can be used to produce the whole leaf protein concentrate (WLPC), which is estimated to contain crude protein level of about 45–60%, which amounts to about 34–45% true protein (Gilani and Lee, 2003). The fibre residue obtained after the protein production is used to feed ruminants, horses, rabbits, or guinea pigs (Lowe, 2002). Moreover, nutrients in LPC are more easily absorbed and digested compared to the original forage crop (Fellows and Hampton, 1992). Phenylhydrazine is a substance used to induce anaemia and blood disorders in man and animals since decades. Phenylhydrazine-

induced toxicity is known to cause lipid peroxidation on the membrane of the red blood cells (RBC), leading to altered haemoglobin referred to as Heinz body, whereby the life span of the erythrocytes is shortened (Ndem *et al.*, 2013). Regardless of the extensive use of *Eremomastax speciosa* (Hochst.) Cufod in traditional medicine, little information is available on the use of the leaf protein concentrate for nutrition and medicinal purposes. Against this backdrop, the present study seeks to provide scientific evidence for the traditional claims on the anti-anaemic properties of *Eremomastax speciosa* (Hochst.) Cufod., using the leaf protein concentrate against phenylhydrazine-induced anaemia in male Sprague-Dawley rats.

MATERIALS AND METHODS

Plant materials

The leaves of *Eremomastax speciosa* (Hochst.) Cufod. were harvested in October 2022 from the University of Nigeria staff quarters, which is located within the Coordinates in degrees and decimal minutes at Latitude: 6°51.4698' N Longitude: 7°23.7462' E. The plant and was identified by Mr. Felix Nwafor of the Department of Plant Science and Biotechnology of the University.

Animal management

A total of 45 male Sprague-Dawley rats, weighing between 130g and 150g, were divided into nine groups of five rats per group. They were obtained from the Department of Animal Health and Production. They were housed in metal cages, in clean environment, and they were provided with clean fresh water and feed *ad libitum*. The research was conducted in accordance with the recommended international guidelines for the care and management of laboratory animals (NRC, 2010), as described in the guidelines of the Institutional Animal Care and Use Committee (IACUC) protocol of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, with approval reference number FVM-UNN-IACUC-2022-0688.

Preparation of the leaf protein concentrate (LPC)

Preparation of plant leaves:

After identification, the leaves and stem of *E. speciosa* were washed and spread in an open room, away from the sun for 3 hours to drip. The leaf protein concentrate was prepared using a modified method of Nwaigwe *et al.* (2022) as follows:

Crushing and pressing of leaves:

Leaves of *E. speciosa* weighing 25kg were washed (wilted for about 40 minutes), cut and grinded, and then homogenized for 30 minutes to separate a large part of the nutritional contents (the dark green juice which is comprised essentially of chloroplasts, cytoplasmic proteins, pigments and vitamins from the indigestible fibre.

Protein coagulation:

The juice was heated rapidly to reach 90°C in 15 minutes, using induction pot, to cause coagulation of the proteins within the pigments, as well as to concentrate soluble vitamins, lipids and minerals in the leaf extract. The green juice was adjusted to pH 8.5 to slow down the action of the plant enzymes and to improve the structure of the coagulum.

Extraction of coagulum:

Thereafter, the coagulated proteins were separated from the whey by filtering through muslin cloth and compressed with mechanical a screw-press to dryness. The coagulum in the form of moist green curd, which contained the leaf proteins concentrate was squeezed out, rinsed with water and then repressed for drying.

Drying and storage:

The extract was then dried at 50°C in a forced-air oven, and stored at 4°C in the refrigerator until it was used for the study.

Experimental design

Forty-five male Sprague-Dawley rats, weighing between 130g and 150g, obtained from the Department of Animal Health and Production, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, were used for the study. The rats were housed in metal cages, in clean environment. The experiment lasted for 28 days, and during an acclimation phase of two weeks, an

ad lib feeding was carried out within the first five days, in order to determine 85% of *ad lib*. Feeding at 85% *ad lib* ensured that leaf protein concentrate (LPC) served as dietary supplement to all the groups. The rats were also given fresh drinking water *ad lib*. Thereafter, the rats were divided into nine groups of five rats per group, and the extract was administered at 100 mg/kg, 200 mg/kg and 400 mg/kg body weight. The standard haematinic used in this study was Ranferon-12®, at the dose of 0.3ml/kg body weight as described Nwaigwe *et al.* (2022). Both the leaf protein concentrate (LPC) of *Eremomastax speciosa* (Hochst.) Cufod. and the hematinic drug were administered using oral gavage to the designated groups, while the controls received distilled water during drenching.

Induction of anaemia

Anaemia was induced through intra-peritoneal (I/P) administration of phenylhydrazine at 40 mg/kg for 2 days, as described previously by (Ndem *et al.*, 2013) Anaemia was confirmed in the induced group following the presence of reticulocytosis and lowered erythron values [PCV (%), HGB (gd⁻) and RBC (x10⁶/L)]. The treatment groups emerged as follows; group 1 (positive control) group 2 (phenylhydrazine only or negative control), group 3 (anaemia + Ranferon®), 4, 5 and 6 were; anaemia +100 mg/kg LPC, anaemia +200 mg/kg LPC, anaemia+400 mg/kg LPC respectively, while groups 7, 8, 9 were; untreated (UT) +100 mg/kg LPC, untreated+200 mg/kg LPC and untreated+400 mg/kg LPC respectively.

Dosage and constituents of Ranferon-12®

The standard hematinic used in this study was Ranferon-12®, manufactured by Ranbaxy® Nigeria Limited, Lagos, Nigeria. It was obtained from a licensed pharmaceutical shop (Elofex pharmacy, Nsukka), and was administered at 0.3ml/kg body weight, as was recommended by Nwaigwe *et al.* (2022). Components of Ranferon -12® include; iron fumarate 305 mg (equiv. elemental Fe 100 mg), folic acid 0.75 mg, cyanocobalamin 5 mg, ascorbic acid 75 mg, zinc sulfate 5 mg, (MIMS.com, 2022).

Haematology and serum biochemistry

About 3mls of blood were collected from each animal using the orbital technique for analysis. Blood samples were collected on day 7 (D0) for baseline data, and three days later, Phenylhydrazine was administered intraperitoneally to the designated experimental groups. Thereafter, blood samples were collected for analysis on days 14 (D14 or induction data), 21 (D21) and 28 (D28). haemoglobin (HGB), platelets, packed cell volume (PCV), red blood cells and white blood cells were determined using the methods of Coles (1986). Part of the blood samples were put in a bottle containing anti-coagulant, Ethylene-diamine-tetra-acetic acid (EDTA), to prevent clotting. The samples were immediately centrifuged at 3400 rpm for 10 minutes to obtain serum, which was carefully aspirated with a Pasteur pipette into sample bottles for the determination of alanine aminotransferase (ALT) and Aspartate aminotransferase (AST).

These were evaluated using Fortress analytical kits (Haematology Triple Pack Box and Clinical Chemistry) obtained from Fortress Diagnostics Ltd, UK. Phyto constituents screening for glycoside, alkaloid, tannin saponin, flavonoids, and cardiac glycosides, was done according to method of Trease and Evans (1983).

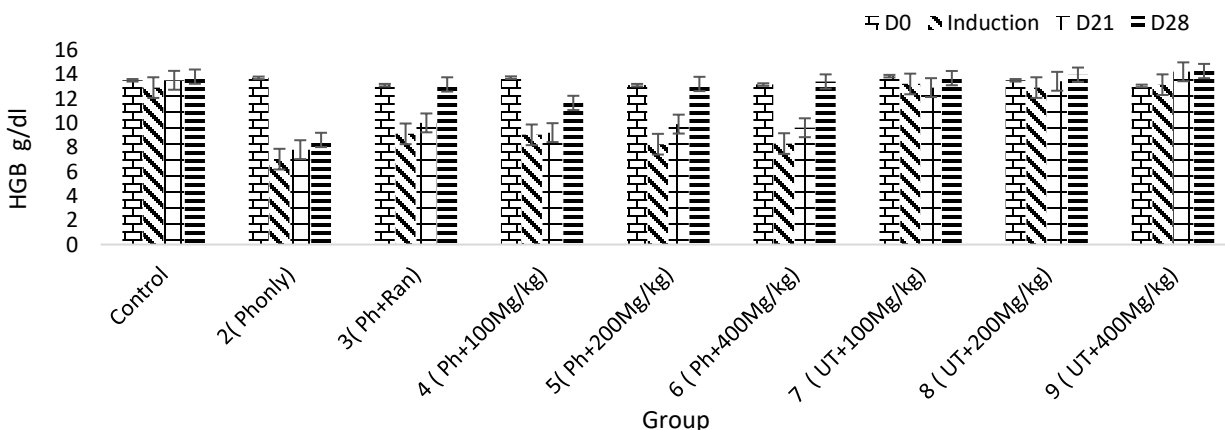
Statistical analysis

Data from this study were analyzed using one-way analysis of variance (ANOVA), and the means separated with Duncan's multiple range test (DMRT). Statistical differences were

considered as significant for p values less than 0.05. Results were presented in tables and histograms, and were expressed in percentages or mean $\pm$ standard error of mean (SEM).

RESULTS

After processing, the leaves of *Eremomastax speciosa* (Hochst.) Cufod. were separated into fibre, LPC and whey. The freshly prepared *Eremomastax speciosa* (Hochst.) Cufod. leaves had a yield of 45% fibre, 50% whey and 5% leaf protein concentrate (LPC), with dry matter contents of 50%, 3% and 40% respectively. The whey gave the highest yield which was about one-half of the entire products, but had the lowest dry matter value, while LPC had the lowest yield. The analysis of phytoconstituents of the leaf protein concentrate of *Eremomastax speciosa* (Hochst.) Cufod. revealed that tannins, saponins, flavonoids, phenols and sterols were positive, while glycosides were negative. In Figure 1, the intra-peritoneal administration of phenylhydrazine significantly ($p < 0.05$) decreased the haemoglobin levels in groups 2-6 compared to the other groups, By D28, the haemoglobin levels of the supplemented groups (4-6) which also received phenylhydrazine treatment showed quicker recovery compared to the negative control which showed significantly ($p < 0.05$) slower recovery (8.6 g/dl) by D28, compared to the rest of phenylhydrazine-treated groups. This was a strong sign of haemolytic anaemia caused by phenylhydrazine treatment.

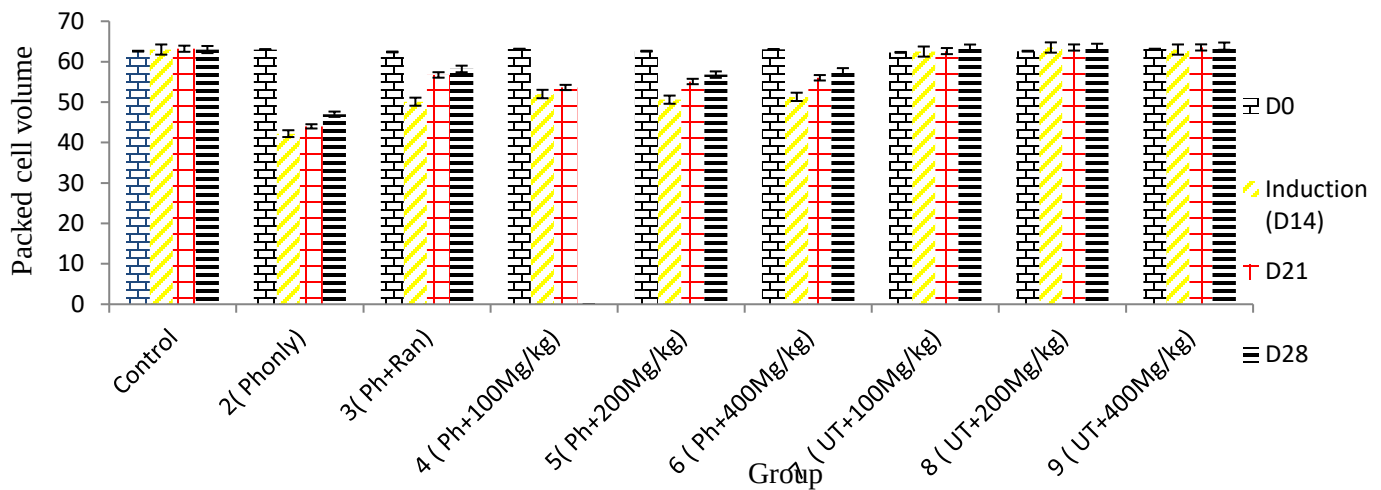


D0= Day 0, D14=Day 14, D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; UT=untreated; Ran = Ranferon.

Figure 1. The haemoglobin values of groups.

The packed cell volume (PCV) of different groups is presented on Figure 2, The negative control was at 42.20% after induction (D14), and increased to 55.44% by D28 which was a sign of

recovery. However, at D28, Groups 3, 4 5 and 6 did not differ significantly ($P>0.05$) from one another, but differed significantly ($P<0.05$) from the positive control, negative control and the untreated groups (UT).



D0= Day 0, D14=Day 14, D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; UT=untreated; Ran = Ranferon.

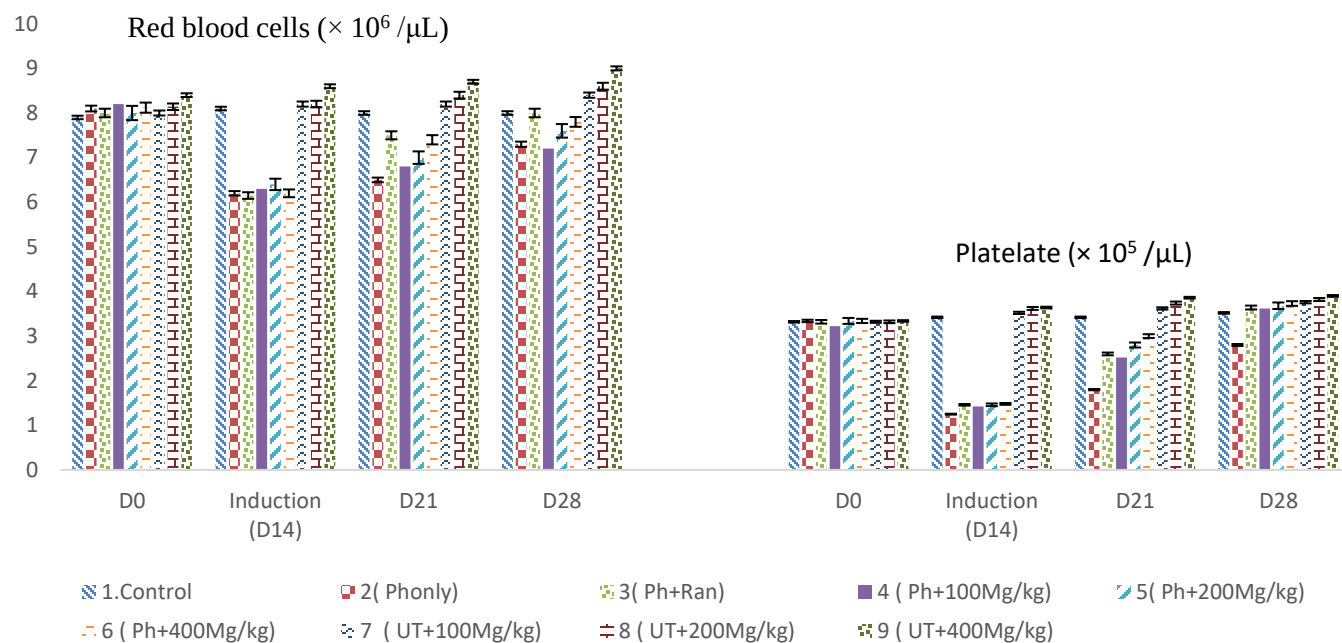
Figure 2. The Packed cell volume of groups.

The red blood cell levels (RBC) and platelet values of the various groups are presented in Figure 3. At D0, the values ranged between 7.9 to 8.4 ($10^6/\mu\text{L}$). After induction and up to D21, the RBC levels in all the phenylhydrazine-treated

groups remained significantly lower ($P<0.05$) compared to the negative control and the other groups. The negative control recovered slowly with a significant decrease ($P<0.05$) of about 47.5% compared to the positive control as well as the supplemented groups (3, 4, 5 and 6).

However, recovery was slower in the negative control group up to D28 compared to the phenylhydrazine-treated groups, whereas the groups supplemented with the *E. speciosa* LPC showed better recovery compared to the negative control (group 2). The platelet values at induction (D14) were significantly ($P<0.05$) reduced in all

phenylhydrazine-treated groups (2-6) compared to the positive control and the untreated groups (groups 7,8 and 9). By D28, the platelet value of the negative group was significantly lower than in the Ranferon group (Group 3) by 23.70%, and for groups 4,5,6,7,8 and 9, the platelet values were lower by 28.65%,23.91%,25.01%, 26.00%, 27.02% and 28.29% respectively.

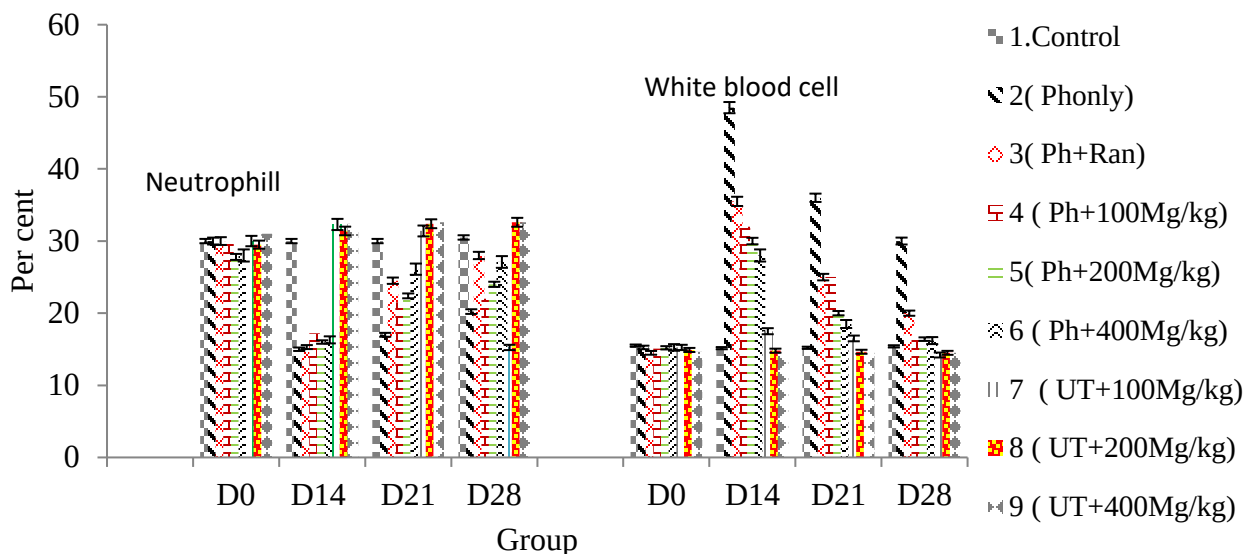


D0= Day 0, D14=Day 14, D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; UT=untreated; Ran = Ranferon.

Figure 3. The red blood cell and Platelet values of groups.

The administration of Phenylhydrazine on D14 (Figure 4), caused a significant decrease ($P < 0.05$) in the Neutrophil values in the negative control and LPC-treated groups compared to the positive control, Ranferon group (Group 3) and the untreated groups (UT) (Groups 7-9). However, between D21 and D28, there was evidence of recovery in all the anaemic groups.

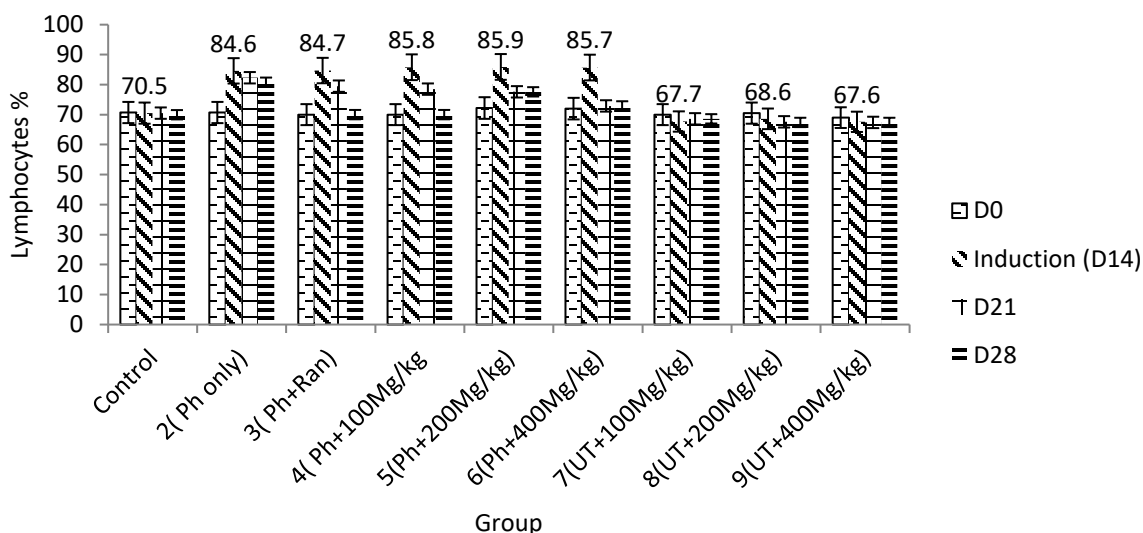
The result of white blood cell values showed that the value of the negative control increased by 68.56%, which was significantly higher ($p < 0.05$) in comparison to all other groups following induction (D14). However, the white blood cell values of the phenylhydrazine-treated groups (3,4,5,6), which were supplemented with *E. speciosa* leaf protein concentrate also showed quicker recovery by D28 compared to the negative control (Group 2).



D0= Day 0, D14=Day 14, D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; UT=untreated; Ran = Ranferon.

Figure 4. The Neutrophil and white blood cell values of groups.

The result of lymphocyte values in Figure 5 showed an increase of about 16.67% during anaemia induction in the negative control compared to the positive control. However, the groups treated with phenylhydrazine and supplemented with *E. speciosa* leaf protein concentrate (LPC) also reverted to normal values by D 28.

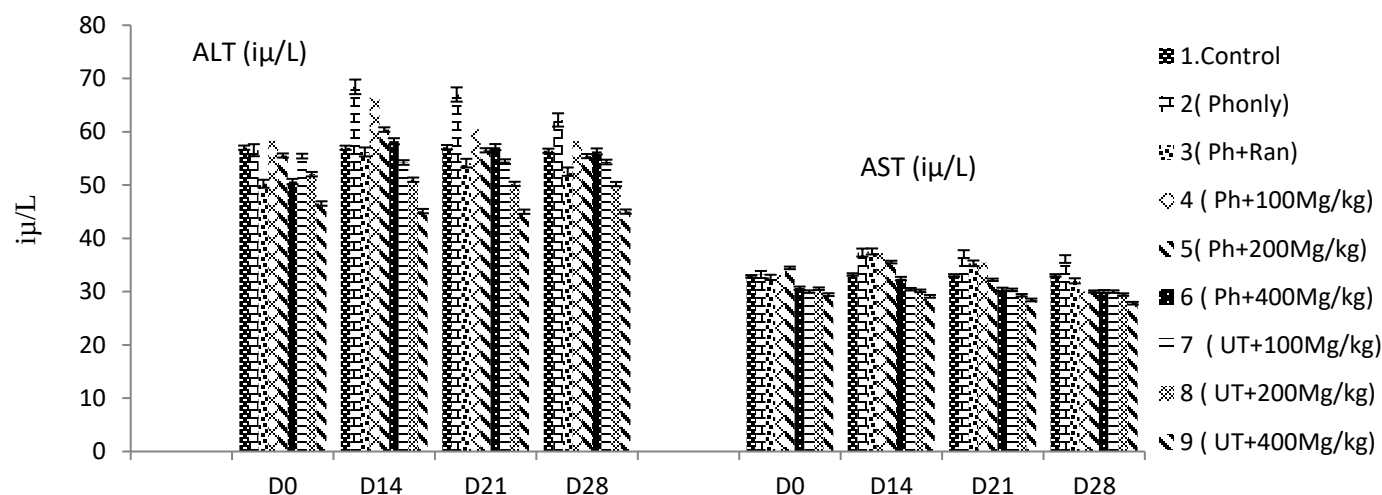


D0= Day 0, D14=Day 14, D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; UT=untreated; Ran = Ranferon.

Figure 5. The lymphocyte values of groups.

Figure 6 shows the Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) values. After induction (D14), the ALT values were significantly increased in the negative control (Group 2) compared to the other groups. Recovery was also faster in these groups (3,4,5 and 6) compared to the negative control, while Group 9 (400mg/kg) had the lowest ALT level of

27.83 μL . The result of the Aspartate aminotransferase also showed that the negative control had significantly ($P<0.05$) highest value of on D 14, followed by the supplemented anaemia group at 100 mg/kg (Group 7), while significantly lowest value ($P<0.05$) of 45.00 μL was also recorded in Group 9 supplemented with 400 mg/kg of the Leaf protein concentrate.



D0= Day 0, D14=Day 14 (Induction), D21= Day 21 D28=Day 28; Ph = Phenylhydrazine; ALT= Alanine amino transferase; (AST) =Aspartate aminotransferase UT=untreated; Ran = Ranferon.

Figure 6. Alanine amino transferase (ALT) and Aspartate aminotransferase (AST) values of groups.

DISCUSSION

Anaemia in invertebrates is a common and serious haematological problem and could be caused by numerous conditions which include malnutrition, drug toxicity, genetic or acquired defects, or blood loss due to parasitic attacks, trauma etc. (Coles, 1986; WHO, 2010; Ndem *et al.*, 2013). Therefore, the intention of this study was to establish scientific evidence on the ability of the leaf protein concentrate of *E. speciosa* to ameliorate induced anaemia in rats using phenylhydrazine. Consequently, anaemia is associated with reduced oxygen-carrying ability of the blood, which commonly occurs as a sign of some disorder rather than a disease (Weiss and Goodnough, 2005). Some of the symptoms

include malaise, pale skin, shortness of breath, headaches, chest pain, and cold hands and feet (FAO *et al.*, 2017).

The anaemic effects of phenylhydrazine have been shown to cause haemoglobin denaturation through an oxidative action (Shukla, 2012; Hariom, 2017), thus reducing the life span of the red blood cells (Jollow and McMillan, 2001; Diallo *et al.*, 2008 and Ndem *et al.*, 2013). Therefore, the application of LPC in this study was very pertinent, considering its high nutritional content (Webb, 2022), as well as its established roles in management and treatment of some diseases including anaemia (Gilani and Lee, 2003; Uyoah *et al.* 2014 and Dais, 2019). According to the reports of Jollow and McMillan (2001), Arylhydrazine, such as phenylhydrazine (PHZ),

dapsone hydroxylamine and divicine can provoke acute haemolytic anaemia in vertebrates. Consequently, this study showed that supplementation with leaf protein concentrate of *E. speciosa* in both the challenged and unchallenged groups resulted in significantly higher erythron values compared to the negative control. This observation agrees with the findings of Ndem *et al.* (2013) who indicated that *E. speciosa* leaf extract showed a similar anti-anaemic activity at 300 mg/kg and 500 mg/kg respectively. According to Roy *et al.* (2023) Aspartate aminotransferase and Alanine aminotransferase show rapidly increased levels when the liver is damaged for reasons such as hepatic cell necrosis, hepatitis, cirrhosis and intoxication with xenobiotics. Alanine amino transferase is a hepatic cytosolic enzyme, quite specific of liver injury, and released into the blood stream when there is liver cell necrosis, while AST is also found in some other organs such as the lungs, heart, skeletal muscle, and kidneys. However, estimated values of the biochemical parameters such as Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) in the rats treated with phenylhydrazine, and supplemented with the leaf protein concentrate of *E. speciosa* revealed a better status of the hepato-renal organs (Dufour *et al.*, 2002; Mbemba *et al.* (2020). Therefore, the results of the present study show that the doses of leaf protein concentrate of *E. speciosa* at (100 mg/kg 200 mg/kg to 400mg/kg) had good effects in both the treated and untreated groups, thus justifying the traditional use of the leaves for the treatment and management of anaemia and other related conditions. However, Siwe *et al.* (2015) indicated that at high doses of between 800-1600 mg/kg, the leaf protein concentrate was no longer protective against anaemia, despite the presence of phyto constituents such as flavonoids and alkaloids. They also reported that animals that received this range of doses (800 to 1600 mg/kg) showed histopathological lesions in organs such as lungs (diffuse alveolar damage) and kidneys (tubular cell necrosis, reduced glomerular space, scattered inflammation), while the liver presented with vascular congestion and biliary stasis. This is an indication that the medicinal use of either the leaf extract or the leaf protein concentrates of *E. speciosa*, could be limited by the dose.

CONCLUSION

This study has shown that considering the doses and duration of use, the leaf protein concentrate of *E. speciosa* was capable of reversing the anti-anaemic effects of phenylhydrazine in the rats under investigation. Therefore, more works could be done to ascertain if it could have deleterious effects on animals, particularly with increased doses and duration beyond the values used in this study.

Conflict of Interest

The authors have no conflict of interest to declare.

Author contribution

CON designed the study, analyzed the work and wrote the first draft of the manuscript with CUN. Data collection and gathering of materials were done by CUN, UCO and LOR. All the authors participated in the review of the manuscript. UCO and LOR provided reagents and performed laboratory analysis, while CON and CUN interpreted the data. All authors read and approved the final version of the manuscript.

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