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Original Research Article

Pharmacological Study of Anti-Anemic Activity of Ethanol Leaf Extract of a Spice Plant (*Piper guineense*) on Phenylhydrazine-induced Anemia in Wistar Rats

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Abstract: Anemia is a disease condition associated with reduced blood levels of red blood cells, hemoglobin, hematocrit and alteration other hematological indices, leading to decreased ability to transport oxygen in the body. It can be precipitated by many risk factors that may occur independently or concurrently. Tradomedicinal practice claims to treat anemia by use of herbal plants which are readily available, accessible and affordable when compared to pharmaceutical drugs. This work therefore sought to pharmacologically study the anti-anemic activity of a spice plant (*Piper guineense*) on phenylhydrazine-induced anemia in wistar rats. This was done by randomizing thirty adult male wistar rats into six groups (1-6) of five animals per group, measuring their hematological indices before (at day 0) and after (at day 7, 14 and 21) induction of anemia with phenylhydrazine (40 mg/kg, i.p.). Groups 1, 2 and 3 respectively served as normal, negative and positive control, while groups 4, 5 and 6 received *Piper guineense* leaf extract at 125, 250 and 500 mg/kg respectively. The results of this study shows that *Piper guineense* leaf extract particularly at medium and high doses, and with respect to negative control, ameliorates phenylhydrazine-induced alterations on hematological indices, by producing significant ($p<0.05$) lesser percentage decrease in blood levels red blood cells, hemoglobin and hematocrit, as well as exhibiting significant ($p<0.05$) lesser percentage increase in blood levels of other hematological indices analysed in this study. The results also indicate that *Piper guineense* leaf extract when compared to positive control, produces insignificant ($p>0.05$) activity, particularly at days 14 and 21. This study provides a robust scientific validation of tradomedicinal claim on *Piper guineense* leaf as anti-anemic agent, therefore recommends further studies that will unveil the bioactive principle(s) and the molecular mechanism/pathway responsible for the observed activity.

Keywords: *Piper Guineense*, Extract, Anti-Anemic, Phenylhydrazine.

INTRODUCTION

Anemia is a hematological disorder that constitutes public health problem on global scale, particularly in the developing and under developed countries [1, 2]. About 24.8% of world population was affected with anemia between 1993 and 2005, with highest prevalence in pre-school children and lowest in men [3]. The prevalence of anemia is also high in Sub-Sahara Africa [4], including Eastern Nigeria [5]. Studies have reported oxidative stress [6], malaria [7], and other conditions such as iron and micro nutrient deficiency, gastrointestinal parasites (hookworms) and HIV [8], as

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causes of anemia in humans. In humans, anemia is characterized by deficiency of well-functioning red blood cells (RBCs) with hemoglobin (Hb) concentration less than 13 g/L in men and 12 g/L in women, manifesting symptoms like pallor, body weakness, exertional dyspnea, confusion, increased cardiac output, and decreased quality of life [9], which are aggravated by oxidative stress [6]. Common treatment of anemia employs oral administration of pharmaceutical iron, but its use for long term therapy poses some problems such as nausea, vomiting, constipation, stomach pain and poor gastrointestinal tract absorption [10]. Poor gastrointestinal tract absorption of iron is attributable to overproduction of a peptide hormone (hepcidin), which plays a critical role in iron homeostasis, leading to inappropriate retention of iron in reticuloendothelial cells, resulting in iron-restricted erythropoiesis [11]. Retention or excess of oral free iron activates Fenton reaction, hence generates free radicals that cause damages to cell membranes, lipids, proteins and nucleic acids, as well as stimulating inflammatory mediators [12].

In view of constraints surrounding the use of pharmaceutical iron and other synthetic compounds in management of anemia in humans, potential use of medicinal plants is recently, a subject of significant interest. Studies have reported anti-anemic potential of several medicinal plants [13, 14]. Medicinal plants present considerable socio-economic advantages related to their folkloric knowledge, availability, accessibility, affordability and environmental friendliness [15].

Among the rich floristic heritage in Nigeria, is *Piper guineense*. It is a perennial spice plant from family Piperaceae. The leaves have peppery taste, and are pale greenish in color when fresh. Among the Igbo tribe in Southern Nigeria, *Piper guineneense* has extensive ethnomedicinal applications in management of many human and animal ailments, including anemia, and also used in blood-boosting. Previous study by [16], on the *Piper guineense* reveals the presence phytochemicals like alkaloids, tannins, flavonoids and saponins, which are associated with various biological activities of plants in general [17]. Some of the reported bioactivities of *Piper guineense* include antibacterial [18], antioxidant [15], hepatoprotective [19], anti-inflammatory [20].

Considering extensive utilization of *Piper guineense* by Igbo ethnic group in Nigeria, our study therefore sought to establish the scientific bases of its ethnomedicinal application in management of anemia.

MATERIALS AND METHODS

Drugs, Chemical and Equipment

Phenylhydrazine hydrochloride (Solag Allied Chemicals, Ltd, Nigeria), Ferrous sulphate (Therapeutic Lab. Nigeria Limited) Tween 80 (Meks Global Ltd, Nigeria), 96% Ethanol (Gungsdong Guandgua Chemical Factory, China).

Animal Handling and Ethics

The animals used in this study included (i) thirty adult male wistar rats that weighed between 200-240 g. (ii) thirteen adult albino mice of both sexes that weighed 18-22 g. The animals were bred and housed at Animal Facility Center of Madonna University, Nigeria. They were adequately fed with standard pellet diet and water *ad libitum.* The animals were denied of food and water for 18 and 2 hours respectively, prior to experiment. The animals were handled in accordance with internationally accepted guideline published by [21]. Ethical approval (MAU/DRC/HD/E/PHARM/2025/101) for this study was granted by Research Ethics Committee of Madonna University, Nigeria.

Collection, Identification and Processing of Plant Material

Fresh leaves of the plant were collected from pest-free farm in Okigwe, South-Eastern Nigeria. The leaves were sent to the laboratory of Plant Science and Biotechnology, University of Port Harcourt, Nigeria, for identification and authentication. The leaves were identified and authenticated as *Piper guineense*, and was assigned voucher specimen number, UPH/P/251. The leaves were garbled to remove extraneous matters (such as the stem and root) and washed with clean tap water. Thereafter, the leaves were spread in a well-ventilated room for 2 weeks for drying at room temperature, to prevent the loss of the essential phytochemicals. The dried leaves were then milled into coarse powder with a milling machine, and stored in a labeled air-tight container until when required.

Extraction

The extraction was done using maceration technique, proposed by [22], with slight modifications About 500 g of the coarsely powdered dried leaves of the plant was weighed and macerated in 2.0 liters of 96% ethanol in an air-tight container to avoid evaporation of extraction solvent. The mixture was intermittently agitated at interval of 6 hours for 72 hours. Thereafter, the mixture was then filtered through Whatmann filter paper into a clean previously weighed empty beaker. The above extraction procedure was repeated. The beaker and its content were kept in a hot air oven at 40˚C until ethanol was expelled, and solid crude residue extract obtained. The final weight of the beaker and its content was determined, and the actual weight of the crude residue extract was calculated by subtracting the initial weight of the empty beaker from the final weight of the beaker and its content. The difference was the weight of solid residue extract (4.37 g)

Acute Oral Toxicity (LD_{50}) Test

A method proposed by [23], was employed which involved two phases that used minimal number of thirteen (13) mice.

Phase I: In this phase, nine (9) mice were used, divided into three groups of three animals. Doses of 10,100 and

1000 mg/kg body weight of extracts were orally administered to group one, two and three animals respectively. Then animals were monitored for signs of toxicity or death for 48 hours.

Phase II: This phase was conducted using the rest of the mice, which were divided into four groups of one animal per group. From the result obtained in phase I, the animal groups, one, two and three were given 1600, 2900 and 5000mg/kg body weight of the extracts respectively and group 4 served as normal control. The animals were monitored 6 hourly for 24 hours for physiological signs of toxicity or death.

Pharmacological Study of Anti-anemic Activity

Evaluation of anti-anemic activity was conducted according to procedure proposed by [24], with slight modification. The procedure is based on blood levels of hematological indices such as red blood cells (RBCs), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBCs), eosinophils (Eos) and basophils (Bas) in rats, before and after induction of anemia.

Induction of Anemia

In this study, anemia was induced in adult male wistar rats by intraperitoneal (i.p) injection of phenylhydrazine hydrochloride at 40 mg/kg daily for two consecutive days [25, 26].

Confirmation of Presence of Anemia

Blood samples from rats injected with phenylhydrazine were collected and analyzed. Rats were considered anemic when the RBCs and Hb contents of blood reduced by 30% relative to normal control [27-29].

Experimental Protocol

Thirty adult male wistar rats that weighed between 200-240 g were distributed randomly into six groups of five animals per group (i.e. n=5). Anemia was induced in groups 2-6, but not in normal control group (i.e. group 1). Thereafter, various animal groups were treated as follows:

- Group 1(normal control) received 10 ml/kg Tween 80 (3% w/v),1 x daily for 21 days, p.o.
- Group 2 (negative control) received 10 ml/kg Tween 80 (3% w/v), 1 x daily for 21 days, p.o.
- Group 3 (positive control) received 5 mg/kg Ferrous sulphate,1 x daily for 21 days, p.o.
- Group 4 (treated with plant extract) received 125 mg/kg, 1 x daily for 21 days, p.o.
- Group 5 (treated with plant extract) received 250 mg/kg, 1 x daily for 21 days, p.o.
- Group 6 (treated with plant extract) received 500 mg/kg, 1 x daily for 21 days, p.o.

Collection and Analysis of Blood Samples for Hematological Parameters

From each rat, blood sample (0.5 ml) was collected into EDTA container on day 0, before induction of anemia, and at 7 days interval for 21 days after induction of anemia. The blood was collected by puncturing the tail vein of the rat, and analyzed using Haem Analyzer (Dymind DH-36)

Analysis of Statistical Data

The data generated in this study were analyzed at 5% alpha level of significance, using one way analysis of variance (ANOVA) followed by Post-Hoc Duncan's Comparison Test. Probability less than 0.05 (i.e $p<0.05$) is considered significant, while probability greater than 0.05 (i.e $p>0.05$) is considered insignificant. Values are presented in the tables as mean ± standard error of mean (SEM).

RESULTS

Result of Acute Oral Toxicity (L_D50) Test of Ethanol Extract of *Piper guineense* Leaf

The result of acute toxicity test in Table 1 shows that acute oral administration of the ethanol extract of *Piper guineense* to mice in both phases causes no physiological change or death within 48hrs. The oral L_D50 the extract in rat is therefore greater than 5000mg/kg.

Result of Anti-anemic Activity

Before induction of anemia, the values of hematological parameters in both control and test groups were within normal range. Injection of phenylhydrazine significantly altered hematological indices (Tables 2-10) of diagnosing anemia. While phenylhydrazine caused significant percentage decrease in the mean values of RBCs, Hb and Hct, it also caused significant percentage increase in mean values of MCV, MCHC, WBCs, Neutrophils, Eosinophils and Basophils. Following the administration of standard drug and extract, we observed significant lesser percentage decrease in blood levels of RBCs, Hb, and Hct as shown in Tables 2-4. Administration of standard drug and extract also produce significant lesser percentage increase in blood values of MCV, MCHC, WBCs, Neut, Eos and Bas as indicated in Tables 5-10.

Table 1: Result of Acute Oral Toxicity Test

Phase	Dose of Extract 1mg/kg	Number of Death
Phase I	10	0/3
	100	0/3
	1000	0/3
Phase II	1600	0/1
	2900	0/1
	5000	0/1
	Negative control	0/1

Table 2: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Red Blood Cells after Phenylhydrazine-induced Anemia in Rats

Red Blood Cells (10^6/uL)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	7.42±0.87	7.51±0.32[a]	1.21↑	7.48±0.10[a]	0.80↑	7.46±0.33[a]	0.50↑
2	7.55±0.65	3.22±0.55	57..35↓	3.80±0.48	49.67↓	3.41±1.04	54.83↓
3	7.48±1.09	4.12±0.63[a]	44.92↓	4.87±0.91[a]	34.89↓	6.66±0.58[a]	10.96↓
4	7.40±0.27	3.62±0.08[a]	51.08↓	3.73±0.27[a]	49.59↓	4.02±0.19[a]	45.66↓
5	7.43±0.090	3.60±0.84[a]	51.55↓	4.11±0.76[ab]	44.68↓	4.75±0.62[a]	36.07↓
6	7.70±0.77	4.21±1.12[ab]	45.32↓	4.68±0.51[ab]	39.22↓	5.83±0.88[ab]	24.29↓

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant with respect to positive control

↑Represents increase relative to initial Red Blood Cells (RBCs) level (ie at day 0) of each group

↓Represents decrease relative to initial Red Blood Cells (RBCs) level (ie at day 0) of each group.

Table 3: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Hemoglobin after Phenylhydrazine-induced Anemia in Rats

Hemoglobin (g/d1)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	13. 24±0.40	13.18±0.88[a]	0.50↓	13.28±0.69[a]	0.30↑	13.20±0.27[a]	0.30↓
2	13.55±0.91	7.28±0.09	46.27↓	7.05±0.57	47.97↓	7.14±0.41	47.31↓
3	13.37±0.76	9.10±0.47[a]	31.94↓	10.25±0.15[a]	23.36↓	11.66±0.64[a]	12.79↓
4	13.42±0.59	7.35±0.61	45.23↓	7.90±0.32	41.13↓	8.04±0.11	40.63↓
5	13.78±0.31	8.10±0.22	41.22↓	8.83±0.56[a]	35.92↓	10..28±0.83[a]	25.40↓
6	13.64±0.75	8.27±0.82[ab]	39.37↓	9.40±0.29[ab]	31.09↓	10.53±0.71[a]	22.80↓

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial Hemoglobin (Hb) level (ie at day 0) of each group.

↓Represents decrease relative to initial Hemoglobin (Hb) level (ie at day 0) of each group.

Table 4: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Hematocrit after Phenylhydrazine-induced Anemia in Rats

Hermatocrit (%)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	41.67±0.34	40.95±0.47[a]	1.73↓	41.44±0.26[a]	0.60↓	42.52±0.30[a]	2.04↑
2	39.75±0.59	25.81±0.18	35.07↓	24.52±0.48	38.31↓	27.86±0.72	29.91↓
3	40. 68±0.80	31.23±0.59[a]	23.23↓	32.02±0.87[a]	21.29↓	35.80±0.19[a]	12.00↓
4	41.50±0.16	27.86±0.20[a]	32.87↓	28.30±0.94[ab]	31.81↓	30.68±0.55[a]	26.07↓
5	39.84±0.07	27.42±0.73[ab]	31.17↓	29.67±0.25[ab]	25.53↓	30.10±0.32[a]	24.45↓
6	41.36±0.45	29.84±0.26[ab]	27.85↑	31.80±0.60[ab]	23.11↓	33.41±0.76[ab]	19.22↓

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial Hematocrit (Hct) level (ie at day 0) of each group.

↓Represents decrease relative to initial Hematocrit (Hct) level (ie at day 0) of each group.

Table 5: Effect of *Piper guineense* Extract and Standard Drug on Mean Corpuscular Volume of Blood after Phenylhydrazine-induced Anemia in Rats

Mean Corpuscular Volume (f1)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	60.71±0.69	59..90±0.45[a]	1.33↓	60.18±0.81[a]	0.9↓	60.56±0.36[a]	0.2↓
2	57.40±0.30	65.72±0.23	14..95↑	65.50±0.37	14.11↑	65.61±0.42	14..30↑
3	57.85±0.26	62.24±0.79[a]	7.59↑	63.19±0.52[a]	9.23↑	61.36±0.11[a]	6.07↑
4	57.08±0.55	65.13±0.18[a]	14.10↑	64.74±0.46[ab]	13.43↑	65.56±0.87[a]	14.86↑
5	58.95±0.14	64.33±0.35[b]	9.13↑	64.80±0.29[ab]	9.92↑	63.89±0.38[a]	8.38↑
6	59..50±0.73	54.87±0.66[ab]	7.78↑	54.42±1.05[ab]	8.54↑	55.03±0.61[ab]	7.51↑

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial MCV (ie at day 0) of each group.

↓Represents decrease relative to initial MCV (ie at day 0) of each group.

Table 6: Effect of *Piper guineense* Extract and Standard Drug on Mean Corpuscular Hemoglobin Concentration (MCHC) of Blood after Phenylhydrazine-induced Anemia in Rats

Mean Corpuscular Hemoglobin Concentration (g/d1)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	31.75±0.31	30.80±0.50[a]	3.00↓	31.16±0.22[a]	1.89↓	32.02±0.46[a]	0.90↑
2	32.47±0.85	33.13±0.52	2.03↑	33.6.9±0.61	3.76↑	34.07±0.72	4.93↑
3	30.86±0.48	30.80±0.17[a]	0.19↓	30.72±0.05[a]	0.45↓	30.55±0.34[a]	1.00↓
4	32.14±0.81	32.20±0.76[ab]	0.20↑	32.17±0.43[a]	0.090↑	32.18±0.52[ab]	0.010↑
5	32.58±0.64	32.60±0.27[ab]	0.060↑	32.49±0.56[ab]	0.27↓	32.25±0.38[ab]	1.01↓
6	31.40±0.85	31..37±0.06[ab]	0.10↓	31.26±0.79[ab]	0.45↓	31.12±0.60[ab]	0.90↓

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial MCHC level (ie at day 0) of each group.

↓Represents decrease relative to initial MCHC level (ie at day 0) of each group.

Table 7: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of White Blood Cells after Phenylhydrazine-induced Anemia in Rats

White Blood Cells (g/d1)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	22.52±0.50	22.71±0.62[a]	0.84↑	22.25±0.26	1.20↓	22.63±0.50[a]	0.50↑
2	23.41±0.79	31.38±0.97	34.04 ↑	33.12±0.35	41.48↑	28.75±0.84	22.81↑
3	24.44±0.76	28. 90±0.17[a]	18.25↑	28.47±0.67[a]	16.49↑	27.85±0.33[a]	13.95↑
4	25.05±0.28	31.84±0.55[a]	27.11↑	30..82±0.73[a]	23.03↑	24.70±0.58[a]	22.55↑
5	23.78±0.63	29.36±0.80[a]	23.47↑	28.06±0.59[ab]	18.00↑	22.70±0.51[ab]	16.48↑
6	25.23±0.07	30.15±0.41[ab]	19.50↑	29.57±0.83[ab]	17.20↑	28.85±0.91[ab]	14.35↑

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial WBC level (ie at day 0) of each group

↓Represents decrease relative to initial WBC level (ie at day 0) of each group.

Table 8: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Neutrophils after Phenylhydrazine-induced Anemia in Rats

Neutrophils (%)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	24.63±0.17	25.00±0.20[a]	1.50↑	24.26±0.74[a]	1.50↓	24.90±0.50[a]	1.10↑
2	24.35±0.48	28.60±0.32	17.45↑	28.25±0.54	16.02↑	27.49±0.15	12.90↑
3	25.29±0.25	28.38±0.66[a]	12.22↑	27.82±0.39[a]	9.81↑	27.75±0.78[a]	9.73↑
4	23.50±0.19	27.61±0.58[a]	17.49↑	27.18±0.18[a]	15.66↑	27.20±0.31[a]	15.74↑
5	25.73±0.83	29.58±0.90[ab]	14.96↑	29.36±0.26[a]	14.11↑	28.71±0.40[ab]	11.58↑
6	24.55±0.42	27.70±0.62[ab]	12.28↑	27.67±0.11[ab]	12.21↑	26.90±0.53[ab]	9.57↑

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial Neutrophils level (ie at day 0) of each group.

↓Represents decrease relative to initial Neutrophils level (ie at day 0) of each group.

Table 9: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Eosnophils after Phenylhydrazine-inducedAnemia in Rats

Eosinophils (%)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	0.90±0.04	0.86±0.60[a]	4.44↓	0.91±0.73[a]	1.11↑	0.89±0.24[a]	1.11↓
2	0.82±0.27	1.09±0.42	32.93↑	1.15±0.30	40.24↑	1.11±0.27	35.37↑
3	0.95±0.81	1.15±0.36[a]	21.05↑	1.05±0.98[a]	10.53↑	1.01±0.83[a]	6.32↑
4	0.79±0.76	1.02±0.77[a]	29.11↑	0.97±0.47[a]	22.78↑	0.98±0.44[a]	24.05↑
5	0.85±0.64	1.00±0.38[a]	17.65↑	0.98±0.19[ab]	15.29↑	0.97±0.31[a]	14.12↑
6	0.88±0.39	1.01±0.51[a]	14.77↑	1.01±0.55[ab]	14.77↑	0.99±0.68[ab]	12.50↑

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial Eosinophils level (ie at day 0) of each group.

↓Represents decrease relative to initial Eosinophils level (ie at day 0) of each group.

Table 10: Effect of *Piper guineense* Extract and Standard Drug on Blood Level of Basophils after Phenylhydrazine-induced Anemia in Rats

Basophils (%)							
Group	**Day 0**	**Day 7**	**%change**	**Day 14**	**% change**	**Day 21**	**% change**
1	0.45±0.50	0.43±0.08[a]	4.44↓	0.46±0.16[a]	2.22↑	0.44±0.12[a]	2.22↓
2	0.52±0.34	0.65±0.65	25.00↑	0.59±0.70	13.46↑	0.56±0.20	7.69↑
3	0.50±0.06	0.58±0.41[a]	16.00↑	0.58±0.45	16.00↑	0.53±0.85	6.00↑
4	0.51±0.53	0.62±0.28[a]	21.57↑	0.62±0.61[ab]	21.57↑	0.62±0.54[a]	21.57↑
5	0.48±0.69	0.57±0.57[a]	18.75↑	0.57±0.74[ab]	18.75↑	0.55±0.68[ab]	14.58↑
6	0.56±0.18	0.64±0.92[a]	14.29↑	0.60±0.53[ab]	7.14↑	0.58±0.07[ab]	3.57↑

Values represent mean ± SEM of n=5, [a]significant ($p<0.05$) with respect to negative control, [b]insignificant ($p>0.05$) with respect to positive control

↑Represents increase relative to initial Basophils level (ie at day 0) of each group.

↓Represents decrease relative to initial Basophils level (ie at day 0) of each group.

DISCUSSION

Study of toxicity of chemicals and herbal plants in animal model is very useful in early identification of adverse effects [30, 31]. A report has established that in addition to mortality, behavioral and physiological changes are also used in determining toxicity in animal model [32]. In this work, the result of acute toxicity (Table 1) reveals that *Piper guineense* extract at 5000 mg/kg did not produce any physiological change or mortality within the period of observation. *Piper guineense* extract up to 5000 mg/kg is therefore non-toxic, which aligns with the report by [23], that LD_{50} equals to, or greater than 5000 mg/kg is considered non-toxic.

For long time now, many experimental studies that involved the use of rats have provided information about diseases and drugs effect on humans. Although there are many animal models of anemia that can be employed in assessment anti-anemic properties of several agents, phenylhydrazine-induced anemia in rats is rapid, reliable and commonly employed model [33]. Based on these advantages, we therefore employed phenylhydrazine model of anemia in wistar rats to pharmacologically study the anti-anemic activity of ethanol extract of *Piper guineense* leaf. Phenylhydrazine is a toxic, non-immunogenic agent that induces anemia characterized by detrimental oxidative stress-mediated mechanisms that include destruction of erythrocyte protein framework, lipid peroxidation, alteration of membrane phospholipids, oxidative degradation of hemoglobin and inflammation [34-37]. Studies have shown that injection (i.p.) of phenylhydazine at 40 mg/kg for two consecutive days induces anemia in rats [33, 34]. In this study, we induced anemia in wistar rat by injecting (i.p) 40 mg kg of phenylhydrazine for two consecutive days. Thereafter, we collected blood samples and determine the levels of RBCs, Hb, Hct, MCV, MCHC, WBCs, Neut, Eos and Bas, which have been reported by [38], as important indices that determine the extent of anemia and efficacy of treatment in experimental animal.

Red blood cells (RBCs) are cell components of blood that play vital role in transportation of oxygen into body tissues. Pathological conditions such as anemia alter RBCs function which may disturb normal functioning of the body. In this study, injection (i.p) of phenylhydrazine to wistar rats caused destruction/hemolysis of RBCs, hence, led to a declined count, which is in line with other reports [33-39]. From the result of this study (Table 2), *Piper guineense* extract significantly ($p<0.05$) counters phenylhydrazine-mediated destruction and improves RBCs count. This finding agrees with reports on other plants with anti-anemic property [40, 41].

Hemoglobin (Hb) performs a vital role as respiratory pigment within RBCs. Hemolysis of RBCs causes release of hemoglobin and accumulation of free heme [42]. In this study, injection of phenylhydrazine (40 mg/kg) into wistar rats caused decreased Hb content of blood, which aligns with other reports [35-39]. A report has shown that phenylhydrazine induces anemia through oxidative stress-mediated denaturation of Hb initiated by free radicals [43], leading to diminished iron absorption [44]. The result of this study (Table 3) reveals that *Piper guineense* exract at 250 and 500 mg/g significantly ($p<0.05$) improves blood level of Hb, suggesting free radicals scavenging/antioxidant-mediated activity. Our earlier in-vitro study had reported free radicals scavenging/antioxidant potential of *Piper guineense* [15].

Hematocrit (Hct) is also known as packed cell volume (PCV). It is a measure of percentage of RBCs contained in the blood. In this study, phenylhydrazine-induced anemia caused decrease in HCT level, which is in agreement with other reports [33-39]. From the result in Table 4, *Piper guineense* extract at 125, 250 and 500 mg/kg significantly reverses the effect of phenylhydrazine on blood Hct level: an action that may be associated with hematoprotective effect of *Piper guineense* leaf extract. This finding corroborates with the reports on another specie of the plant, *Piper betel* [45, 46]. More so, since Hct is a measure percentage of RBC in the blood, increase in RBC may have led to increase in Hct.

Mean corpuscular volume (MCV) is a measure of average size of RBCs, while mean corpuscular hemoglobin concentration (MCHC) is a measure average concentration of Hb in a RBC. In this study, phenylhydrazine-induced anemia caused an increase in MCV and MCHC levels, which is in conformity with other studies [47, 48]. Report has indicated that hemolytic effect of phenylhydrazine on RBCs prompts a compensatory increase in size and concentration of the remaining unhemolysed RBCs [49]. From the result in Tables 5 and 6, *Piper guineense* extract significantly reduces phenylhydrazine-induced rise on MCV and MCHC levels. This may be attributable to antioxidant-rich constituent, which may have facilitated normalization of RBCs size and Hb concentration. This finding agrees with report on other anti-anemic plants like *Malus domestica* (apple vinegar) [50], and *Limonia acidissima* [51].

Phenylhydrazine-induced anemia in this study witnessed rise in WBCs level, which is similar to observation made by other studies [47, 48]. The rise is associated with activation of immune system, leading to increase in number of circulating leukocytes, including WBCs [52]. From the result (Table 7), *Piper guineense* extract significantly began to normalize the WBCs level, starting from day 7. Therefore we hypothesize that the ability of *Piper guineense* extract to normalize the phenylhydrazine-induced elevation of WBCs level may be on the account of immunomodulatory activity of the plant, mediated via attenuation of oxidative stress and/or free radicals scavenging.

Phenylhydrazine injection into rats in this study caused an increase in neutrophils count, which is in agreement with finding by [53], that increase in neutrophils count occurs as a response to inflammatory state caused by hemolytic crisis, hence activating immune response. Also, elevated neutrophils count following phenylhydrazine-induced anemia may suggest an attempt by the body to clear damaged RBCs and other cellular debris. The result in table 8 shows that *Piper guineense* extract at various doses (125, 250 and 500 mg/kg) began from day 7 to mitigate the inflammatory state induced by phenylhydrazine, hence pointing at *Piper guineense* as potential protective agent against anemia.

This study observed increase in eosinophils and basophils counts following administration of phenylhydrazine into rats. This may be due to phenylhydrazine-induced inflammation, because it is reported that increase in eosinophils and basophils counts are associated with inflammation/allergic reaction [54-56]. Result in Tables 9 and 10 shows the ability of *Piper guineense* extract at various doses to significantly normalize the elevated eosinophils and basophils counts, hence, suggesting anti-inflammatory potential of the plant. Our previous in-vitro study reported anti-inflammatory potential of *Piper guineense* [20]. More so, since eosinophils and basophils are type of WBC, normalization of WBC count may have led to normalization of eosinophils and basophils count.

CONCLUSION

In this study, intraperitoneal injection of phenylhydrazine at 40 mg/kg for two consecutive days induced anemia characterized by alteration on hematological indices in wistar rats. *Piper guineense* extract, particularly at 250 and 500 mg/kg ameliorates the phenylhydrazine-induced alteration on hematological indices, hence possessing anti-anemic activity that can be comparable to standard drug (Ferrous sulphate) used in this work. The present study did not determine the active principle(s) and exact molecular mechanism/pathway responsible for anti-anemic activity of *Piper guineense*, therefore we recommend further researches to undertake these aspect.

Conflict of Interest: The authors have no conflicting interest

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