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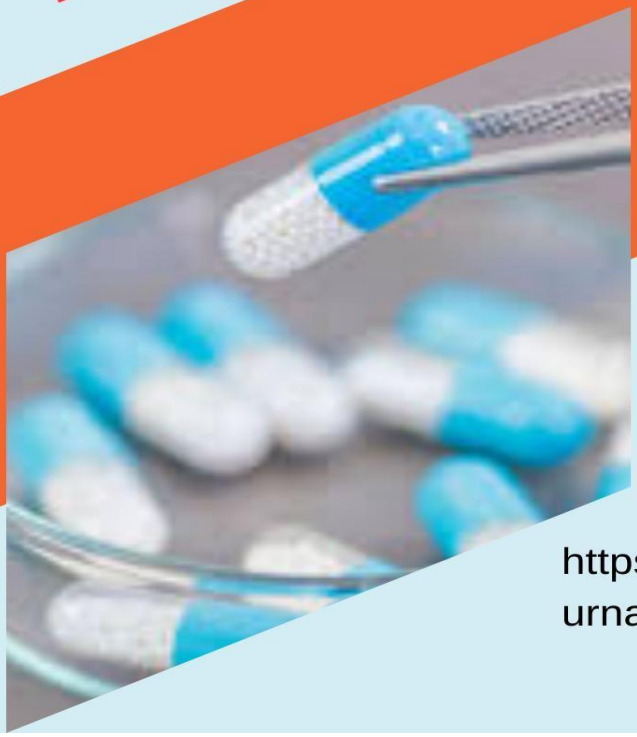

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Phyllanthus niruri Linn Aqueous Extract's Antianaemic Effect in 2, 4-Dinitrophenylhydrazine-Induced Anaemia in Rats

Wei Deng , Xie , gyang Chi

Department of **Pharmacological Research**

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Abstract

Background: An annual plant called Phyllanthus niruri has long been used in Asia and Africa to treat a variety of illnesses, including jaundice, asthma, hepatitis, flu, dropsy, diabetes, malaria, hemorrhages, diarrhea, and anemia.

Objective: The haemotological and biochemical characteristics of P. niruri in 2,4- initrophenylhydrazine-induced hemolytic anemia in Wister rats are assessed in this study for potential antianemic effects.

Methods: This study assesses the plant extract's antianaemic activity in Wister rats with 2,4-dinitrophenylhydrazine-induced hemolytic anemia using body weight, hemotological, and biochemical data. Folic acid was utilized as a positive control, and the plant extract was given in three separate doses (250, 500, and 1000 mg/kg b.wt). PCV, Hb, RBC, WBC, MCV, MCH, and reticulocyte count were among the haematological parameters that were assessed. Bilirubin (total and unconjugated) and oxidative stress indicators (MAD, catalase, and SOD) were also examined.

Results: The findings revealed that the concentrations of PCV, Hb, and RBC increased significantly ($P<0.001$) at doses of 500 and 1000 mg/kg b.wt, respectively, while there was no significant ($P>0.05$) effect at a dose of 250 mg/kg b.wt. The concentrations of WBC, MCV, MHC, and reticulocytes decreased significantly ($P<0.001$) at doses of 500 and 1000 mg/kg b.wt.

Conclusion: The findings imply that the P. niruri plant's aqueous whole plant extract is comparatively safe and has a strong anti-anaemic effect; as a result, it might be a promising lead in the development of a medication to treat hemolytic anemia.

Keywords: Phyllanthus niruri; antianaemic; 2,4-dinitrophenylhydrazine; indicators of oxidative stress; hemolytic anemia

Introduction

One of the prevalent blood-related conditions that affect people worldwide is anemia [1]. Although it can appear at any stage of life, it primarily affects small children and pregnant women [2]. Because malaria and other parasite illnesses are more common in the tropics and can lower hemoglobin levels or circulating red blood cells, anemia is

more common there [3]. The World Health Organization (WHO) classifies anemia in 69 nations as a terrible public health issue (prevalence > 40%) for children under five as well as for expectant mothers in 68 nations. The National Family Health Survey (NFHS-3) reports that the prevalence of anemia is 71% in the developed world, 84% in the developing world, and 79% worldwide [4].
Low iron levels are the main cause of anemia. However, there are additional causes of anemia, including drug toxicity, malaria, parasite infections, dietary inadequacies, and genetic or acquired defects [5]. Other names for Phyllanthus niruri include "Chanca piedra" in Spanish, "quebrapiedra" (stone breaker) in Brazil, and Pitirishi or Budhatri in India [6]. In Nigeria, it was referred to as Iyin-olobe by the Yorubas in the southwest, Egi kpeye kpezuma by the Nupes in the north, and Ngwu ite kwowa nasu by the Igbos in

the southeast [7]. In Asia, Africa, and South America, P. niruri is widely used [8] to treat a variety of conditions, including jaundice, asthma, hepatitis, flu, dropsy, diabetes, malaria [9], hemorrhages, diarrheas, dysentery, jaundice, cough, and anemia. It is also said to be effective in treating bronchitis, anemia, leprosy, asthma, urinary tract infections, stimulating the liver, improving digestion, increasing appetite, and producing laxative effects [10]. Additionally, Phyllanthus niruri has historically been used to treat syphilis [8], gonorrhea, tumors, vaginitis, anemia [7], and TB. The pharmacologically active substances found in P. niruri include flavonoids, anthraquinones, alkaloids, saponins, steroids, tannins, and terpenoids, according to a study by Okoli et al. (2010) [8]. It was also discovered that lignans and coumarins were present [6]. By preventing CCl4-induced lipid peroxidation in rats, Harish et al. (2006) demonstrated the high antioxidant potential of P. niruri extracts (aqueous and methanolic) in vivo. As a result, the plant is utilized all over the world to cure a variety of illnesses, including anemia.

Materials and Methods Drugs and Reagents

Folic acid (Emzor Pharmaceutical Industry, Lagos) and 2,4-dinitrophenylhydrazine (British Drug House, UK). Additional analytical-grade chemicals were acquired from BDH, Poole, UK.

Plant Material and Extraction

In October 2018, fresh P. niruri entire plants were gathered from Minna in Niger state, Nigeria. A pharmacognosist from Usmanu Danfodiyo University Sokoto's Department of Ethnomedicine and Pharmacognosy, Faculty of Pharmaceutical Sciences, recognized the plant. A voucher number (PCG/UDUS/Euph/0011) was received and a specimen was placed in the faculty herbarium.

After a thorough water treatment, the plant was dried to a consistent weight. The herb was pounded into a coarse powder using a pestle and mortar. 200g/L was obtained by dissolving 2 kilogram of the powdered plant in 10 liters of distilled water using the cold maceration method. The homogenate was stirred every day for three days at 40˚C. After that, a muslin cloth and Whatman No. 1 filter paper were used to filter the mixture. After being condensed to dryness using a freeze drier, the filtrate was kept at 4˚C until the study needed it.

Animals Grouping and Experimental Procedure

The study employed adult Wister albino rats, both male and female, weighing between 180 and 200 grams and approximately 12 weeks of age. They came from A.B.U. Zaria, Nigeria's Faculty of Pharmaceutical Sciences. Before the experiment began, the animals were kept in standard cages at the animal house of the Faculty of Pharmaceutical Sciences at Usmanu Danfodiyo University Sokoto. They were given optimal feed (standard commercial diet from Vital Feeds company, Jos, Plateau State, Nigeria) and full access to drinking water for two weeks. Using computer-aided randomization (Decision Analyst Stats 2.0), the animals were split into different groups at random, and each rat was given a permanent marker for simpler identification. A feeding cannula was used to provide the extract orally. To calculate the proper dose in mg/kg body weight, each rat was weighed before

the extract was administered. The upkeep and management of the he animals complied with the Institutional Animal Ethics Committee's rules. Throughout the trial, the rats' weights were tracked.

Induction of Anaemia using 2,4-dinitrophenylhydrazine

In this investigation, a modified approach outlined by Berger (2007) was employed. Three weeks were spent conducting the experiment. Five groups of eight animals each were randomly selected from among the animals (n=8). Using a feeding canula, 2,4-DNPH (20 mg/kg b.wt, p.o.) was given to each animal once a day for seven days in a row. On the eighth day, each rat's tail vein was punctured to obtain a blood sample, which was then placed in heparinized capillary tubes for hematological examination. For the investigation, rats with a PCV drop of at least 30% were deemed anemic [5].By drawing blood from a small incision around the tail and letting the blood pass through a capillary tube to more than two-thirds of the tube, PCV was measured both before and after anemia was induced. After that, a Bunsen burner was used to seal off the tube's end. The samples were centrifuged at 3,000 rpm for five minutes. The PCV

was calculated by placing the capillary tubes on the microhaematocrit. As soon as anemia was established, treatment with the extract began. The treatment groups received varying amounts of plant extract (250, 500, and 1000 mg/kg), the positive control group received 75µg/kg of folic acid, and the negative control group received no treatment. Using an oral feeding cannula, all therapies were given once a day for 14 days in a row.

Haematological Assay

Following a two-week course of treatment with the aqueous extract, cardiac punctures were used to draw blood samples, which were then placed in EDTA vials to prevent clotting. Haematological parameters (RBC, PCV, Hb, WBC, MCHC, MCV, and reticulocyte count) were determined using the samples. With the exception of reticulocytes, all haematological parameters were measured automatically using a Sysmex machine (Model No. KX-21N) manufactured by Sysmex Laboratories Ltd. in the United States. After being centrifuged or combined, the blood samples were run through an apparatus known as an Automated Analyzer, which counts the various cell types and quantities in the blood. The visual approach was used to count reticulocytes [20].

Lipid peroxidation assay (Thiobarbituric acid method) Principle

MDA react with thiobarbituric acid (TBA) to form an MDA-TBA2 adduct that absorbs strongly at 532 nm.

Procedure

As described by (David et al., 1990) 150µL of the sample was diluted to 500µL with double deionized water. In test tubes, 250µL of 1.34% Thiobarbituric acid was added followed by the addition of equal volume of 40% trichloroacetic acid. The mixture was well shaken and incubated for 30 minutes in boiling water (temperature $>90^0$C). Tubes were allowed to cool down to room temperature and the absorbance of Malondialdehyde (MDA) formed was read at 532nm using 0 concentration as blank. The concentration of Malondialdehyde formed was calculated using the following formula.
MDA (µmol/L) = Absorbance at 532 x 1000/1.56.

Enzymatic Assay for Superoxide Dismutase (Kakkar *et al.*, 1984) Principle

The principle of this method is based on the competition between the pyrogallol autoxidation by $O2^{\bullet -}$ and the dismutation of this radical by SOD.

Procedure

Test tubes were labeled as the test and blank and 0.1mL of Buffer was added into test labeled tubes while 0.15mL

was added into blank labelled test tube. 0.83mL of distilled water was then added to both blank and test. Serum (0.05mL) was added into test and incubated at 24^0C for 10 minutes then transferred into cuvette. 0.02mL of Pyrogallol was added to both blank and test. The mixture is immediately mixed by inversion and change in absorbance at 240nm was recorded after 3 minutes approximately. The change in absorbance at 420nm/minute is calculated using the maximum linear rate for both test and blank.

% inhibition = Abs at 420nm /min of blank –Abs at 420nm/min of sample x 100
Abs 420nm/min of blank
Abs – Absorbance

The number of units of SOD is calculated using the following formula:
% inhibition / (100-% inhibition) = Units/ml
One unit of SOD activity was defined as the amount of enzyme that reduces the rate of auto-oxidation of pyrogallol by 50% at standard assay conditions.

Bilirubin Principle

Bilirubin reacts with diazotized Sulphanilic acid to form a colored azobilirubin compound. The unconjugated bilirubin coupled with the Sulphanilic acid in d presence of caffeine produces a colour complex also. The intensity of the color is directly proportional to the concentration of Bilirubin in the specimen and is measured at 546 nm.

Total Bilirubin Procedure

As described by (Olukunle *et al*.,2010). Test tubes were labeled as blank and sample 200µL of reagent 1 was added to both sample and blank tubes followed by the addition of 50µL of reagent 2 to sample test tubes. Reagent 3 (100µL) was added to both sample and blank tubes followed by addition of 200µL of the sample (serum) to both sample and blank tubes. The mixtures were incubated at 20-25^0C for 10 minutes followed by the addition of 1000µL of reagent. The content of the tubes were mixed and incubated at 25^oC for 5-30minutes. The content of the tubes was transferred into a Cuvette and absorbance was recorded at 546nm. The concentration of total Bilirubin was calculated using the following formula.
Total bilirubin (µm/L) = 185 X Absorbance of total bilirubin (546nm)

Direct Bilirubin

Test tubes were labeled as blank and sample, 200µL of reagent 1 was pippetted into both blank and sample test tubes. Followed by the addition of 50µ L of reagent 2 into sample test tubes. Addition of 0.9% NaCl (200µL) was done to both sample and blank test tubes followed by the addition of 200µL of the test sample (serum) into both sample and blank test tubes. The content of the tubes was mixed and incubated for 10min at 20-25^0C. Absorbance was taken at 546nm. The concentration of direct Bilirubin was calculated using the following formula.

Direct bilirubin (umo/L) = 246 X Absorbance of direct bilirubin (546nm)

Statistical Analysis

The Data were expressed as mean ± standard error and were analyzed using One-way Analysis of Variance (ANOVA). The comparison between untreated and Treatment groups was done using the Dunnet's multiple comparison tests. The level of significance was set at $P < 0.05$.

Results

Following the induction of anaemia, body weights of the rats pre and post induction of anaemia with PHZ was evaluated. There was a significant ($P<0.05$) reduction in weight in the PHZ induced animals and a significant ($P<0.01$) increase in weight the groups with various concentration of aqueous extract of *P. niruri* when compared with the untreated group as seen in Figure 1 below.

200

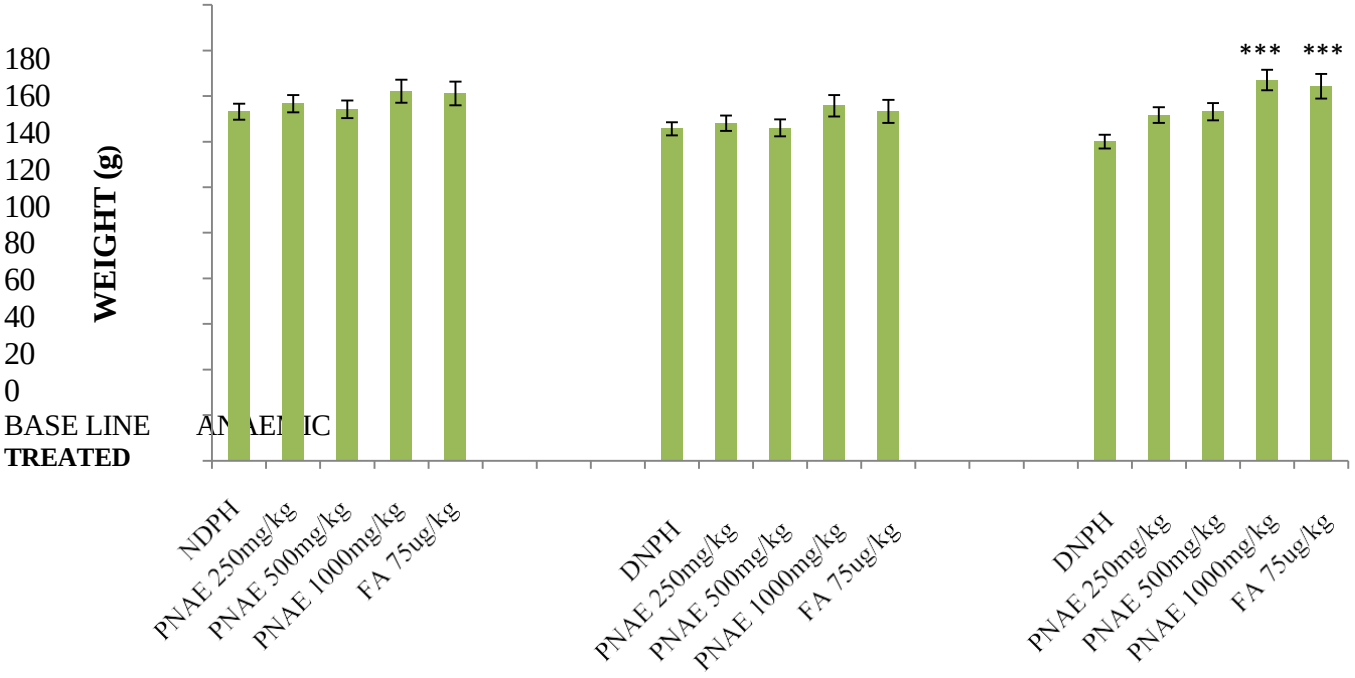


Figure 1: Effect of *P. niruri* Aqueous Extract on weight of Rats pre and post induction of anaemia 14 days after treatment with PNAE. Values represent the means ± SE (n= 8 rats each for all the groups). P<0.05 and P<0.001 was considered significant when compared with untreated group. PNAE = *P niruri* aqueous extract, DNPH = 2, 4-dinitrophenylhydrazine, FA = Folic acid.

Following induction of anaemia treatment, the result of PCV shows a significant (P>0.001) decrease in PCV in PHZ treated groups (at day 7) but a dose-dependent increase in PCV was observed after 14 days of treatment with *P. niruri* extract when compared with the untreated control group compared with the untreated normal control group as illustrated in Figure 2 below.

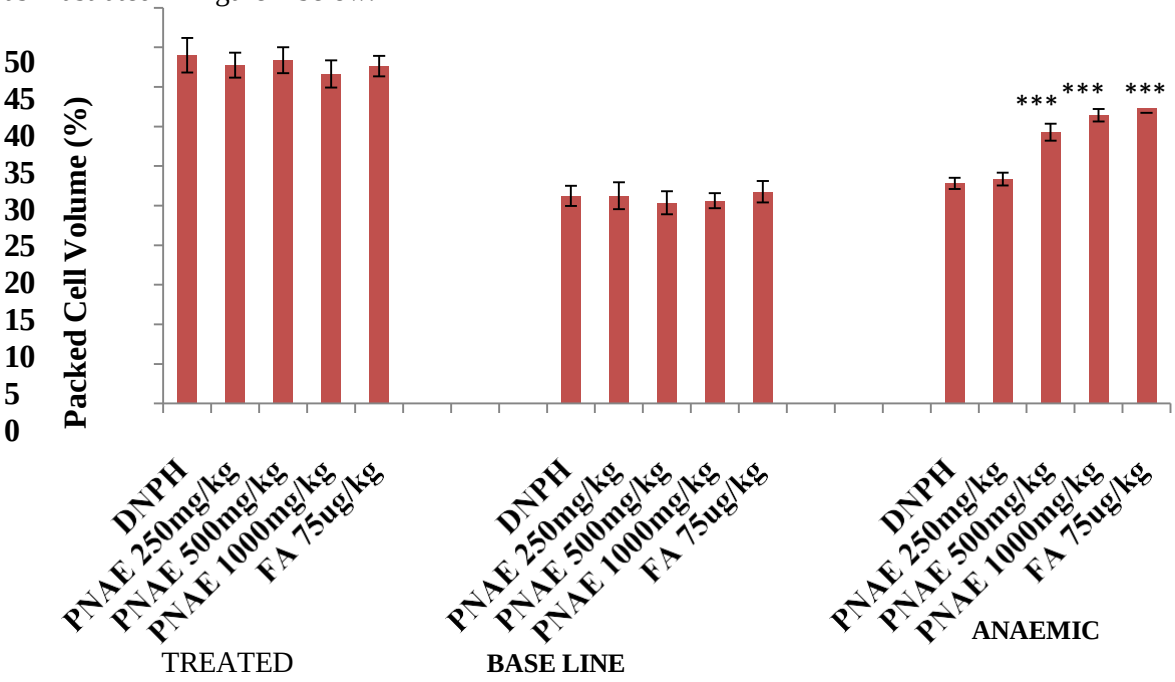


Figure 2: Effect of *P. niruri* aqueous extract on PCV in 2,4-DPHZ-induced anaemic rats at baseline, after induction of anaemia and after 14 days treatment with PNAE. Values represent the means ± SE (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's posttest was used to arrive at the P values. * signifies P<0.05 when compared with untreated group. PNAE = Phyllanthus niruri aqueous extract, DNPH = 2,4-dinitrophenylhydrazine, FA = Folic acid.

The treatment of PHZ induced anemic rats with PNAE for 14 days shows a significant (P>0.001) and dose-dependent increase in Hb concentration and also a significant (P<0.001) decrease in the untreated control group as illustrated in the Figure 3 below.

The result of RBC concentration following treatment of PHZ induced anemic rats with PNAE Produces a significant (P<0.001) and dose-dependent increase in RBCs when compared with the untreated group. There was also a significant (P<0.001) decrease when PHZ group was compared to the control after treatment as shown in Table 1 below.
The result of WBC concentration following treatment of PHZ induced anemic rats with PNAE Produces a significant (P<0.02) and dose-dependent decrease in WBCs when compared with the untreated group. There was also a significant (P<0.001) increase in WBC level when PHZ group was compared to the control after treatment as shown in Table 1 below.

Table 1: Effect of treatment of anemic rats with aqueous extract of *P. niruri* on HB, RBCs, and WBCs

Treatments	**Indices**		
Dose (mg/kg)	HB (g/dl)	RBC ($\times 10^6$µl)	WBC ($\times 10^3$µl)
DNPH PNAE	7.79±0.34	3.19±0.29	19.16±0.38
250	8.21±0.27^{ns}	3.51±0.21^{ns}	17.58±0.24*
500	10.43±0.54*	4.83±0.27*	12.65±0.44*
1000	11.31±0.28*	5.33±0.10*	11.81±0.32*
Folic acid (7.5µg/100g)	11.55±0.36*	5.74±0.16*	10.95±0.39*

Values are expressed as mean ± SEM, n=8. P<0.05 is considered as significant. Values with * are significant when compared to untreated groups.

Also, a significant (P<0.05) decrease in MCV, MCH and reticulocytes was observed at 500 and 1000mg/kg after 14 days treatment with 500 and 1000mg/kg body weight when compared to the 2,4-DNPH induced anemic group of the extract. No significant (P>0.05) effect was observed at a dose of 250 mg/kg body weight as shown in Table 2 below.

Table 2: Effect of treatment of anemic rats with aqueous extract of *P. niruri* on MCV, MHC, and Retics

Treatments	**Indices**		
Dose (mg/kg)	MCV (fl)	MHC (pg)	RETICULOCYTES (%)
DNPH PNAE	93.50±7.14	25.38±1.78	8.30±0.36
250	84.54±3.69^{ns}	23.06±0.79^{ns}	7.44±0.30^{ns}
500	71.59±1.78*	21.61±0.36*	5.67±0.35*
1000	68.63±0.598*	21.00±0.24*	5.35±0.26*
Folic acid (7.5µg/100g	65.35±1.61*	20.05±0.53*	5.11±0.29*

Values are expressed as mean ± SEM, n=8. P<0.05 is considered as significant. Values with * are significant when compared to untreated group.

Biochemical Parameters Oxidative Stress Markers Malondialdehyde

The result of malondialdehyde following treatment of PHZ induced anemic rats with PNAE produces a significant (P<0.05) and dose-dependent decrease in malondialdehyde when compared with the untreated group (PHZ). There

was also a significant (P<0.001) increase in MDA level when PHZ group was compared to the control after treatment as shown in Figure 3 below.

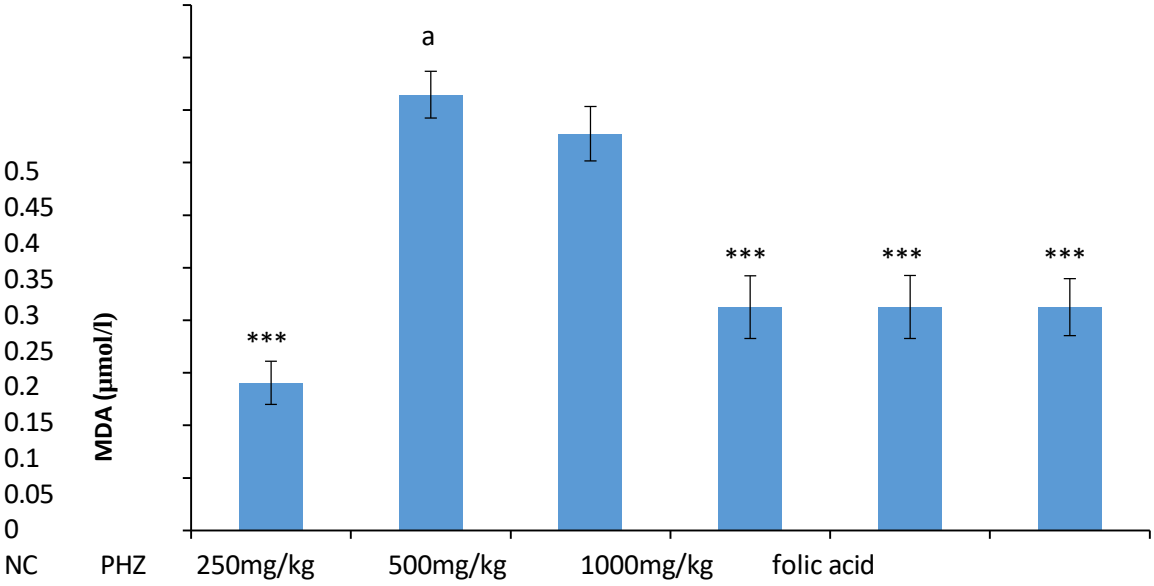
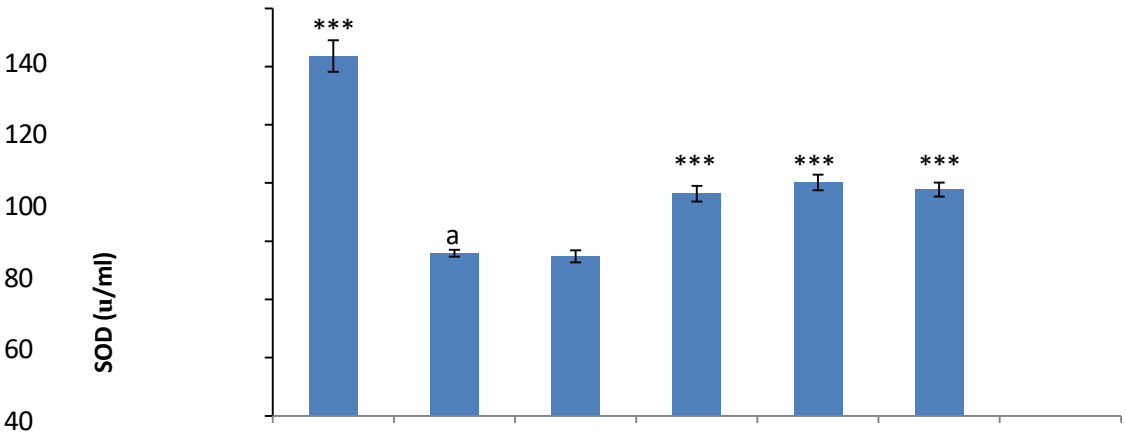


Figure 3: Effect of PNAE on MDA in PHZ-induced Anaemic Rats after 14 days treatment with PNAE. Values represent the means ± SD (n= 8 rats each for all the groups). Oneway ANOVA with Dunnet's post-test was used to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ-treated group while [a] signifies P<0.001when compared with normal control.

Superoxide Dismutase Stimulatory Activity of PNAE

The result of SOD activity following treatment of PHZ induced anemic rats with PNAE shows a significant (P<0.01) and dose-dependent increase in SOD activity when compared with the untreated group (PHZ). There was also a significant (P<0.001) decrease in SOD activity level when PHZ group was compared to the control after treatment as shown in Figure 4 below.



20

0
NC PHZ 250mg/kg 500mg/kg 1000mg/kg Folic acid

Figure 4: Effect of PNAE on SOD in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Values represent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post- test was used

to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ- treated group while [a] signifies P<0.001when compared with normal control.

Effect of treatment on catalase activity

The result of Catalase activity following treatment of PHZ induced anemic rats with PNAE shows a significant (P<0.01) and dose-dependent increase in catalase activity when compared with the untreated group (PHZ). There was also a significant (P<0.001) decrease in catalase activity when PHZ group was compared to the control after treatment as shown in Figure 5 below.

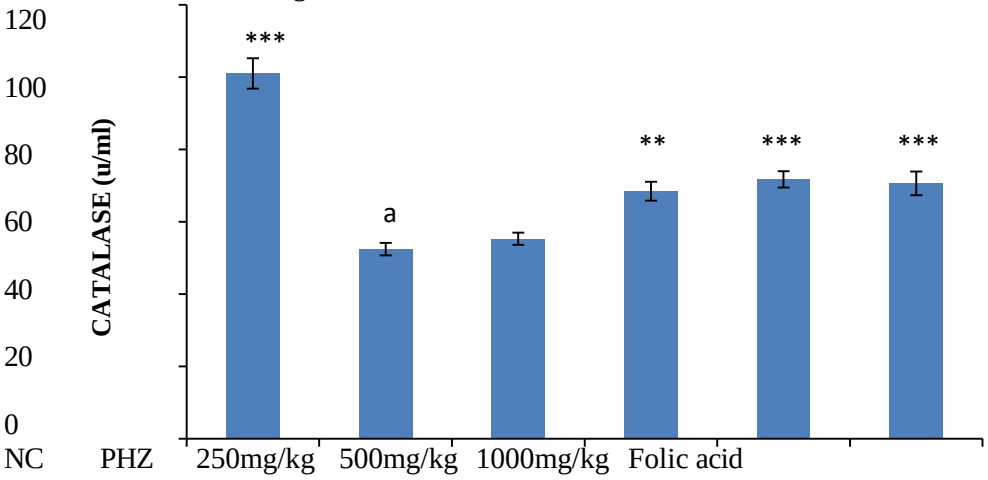
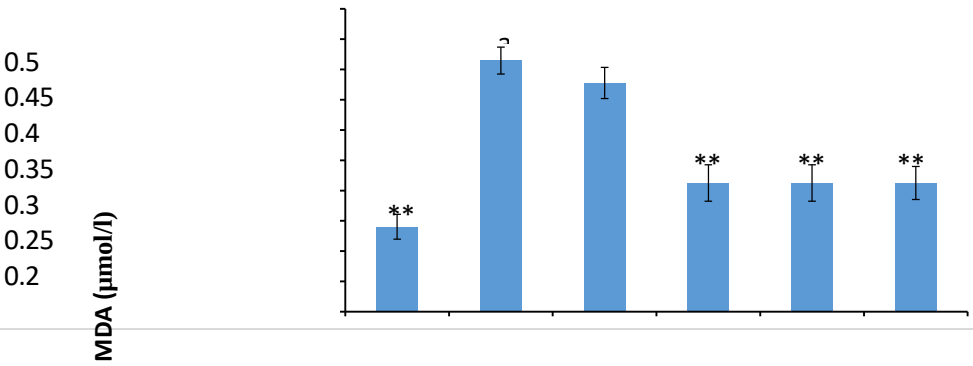


Figure 5: Effect of PNAE on Catalase in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Values represent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post-test was used to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ- treated group. while [a] signifies p<0.001when compared with normal control.

Biochemical Parameters Oxidative Stress Markers Malondialdehyde

The result of malondialdehyde following treatment of PHZ induced anemic rats with PNAE produces a significant (P<0.05) and dose-dependent decrease in malondialdehyde when compared with the untreated group (PHZ). There was also a significant (P<0.001) increase in MDA level when PHZ group was compared to the control after treatment as shown in Figure 6 below.



0.15
0.1
0.05
0
NC PHZ 250mg/kg 500mg/kg 1000mg/kg folic acid

Figure 6: Effect of PNAE on MDA in PHZ-induced Anaemic Rats after 14 days treatment with PNAE. Values represent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post-test was used to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ- treated group while [a] signifies p<0.001when compared with normal control.

Superoxide Dismutase Stimulatory Activity of PNAE

The result of SOD activity following treatment of PHZ induced anemic rats with PNAE shows a significant (P<0.01) and dose-dependent increase in SOD activity when compared with the untreated group (PHZ). There was also a significant (P<0.001) decrease in SOD activity level when PHZ group was compared to the control after treatment as shown in Figure 7 below.

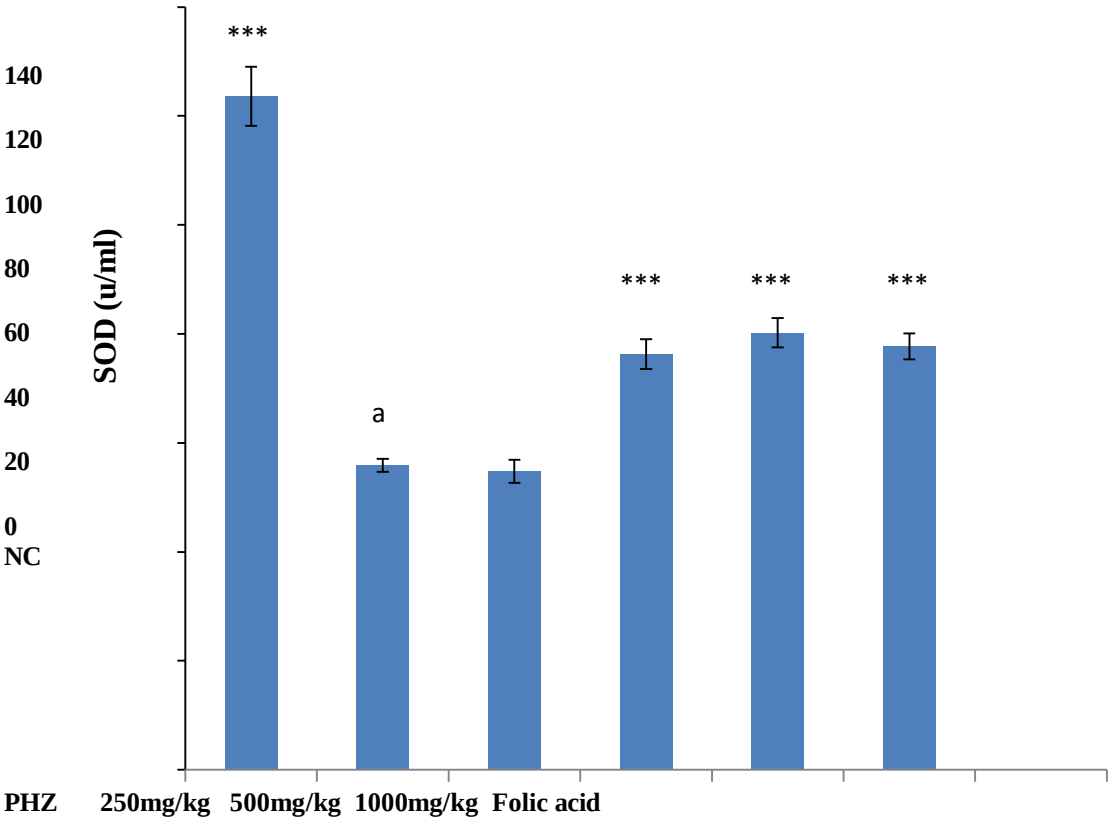


Figure 7: Effect of PNAE on SOD in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Values represent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post-test was used to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ- treated group. While [a] signifies P<0.001when compared with normal control.

Effect of Treatment on Bilirubin

The result of bilirubin assay revealed a significant (P<0.001) and dose-dependent reduction in total, direct and

unconjugated bilirubin when PHZ induced anaemic rats were treated with PNAE compared with the untreated group. There was also a significant (P<0.001) increase in bilirubin when PHZ group was compared to the control after treatment as shown in Figure 8 below.

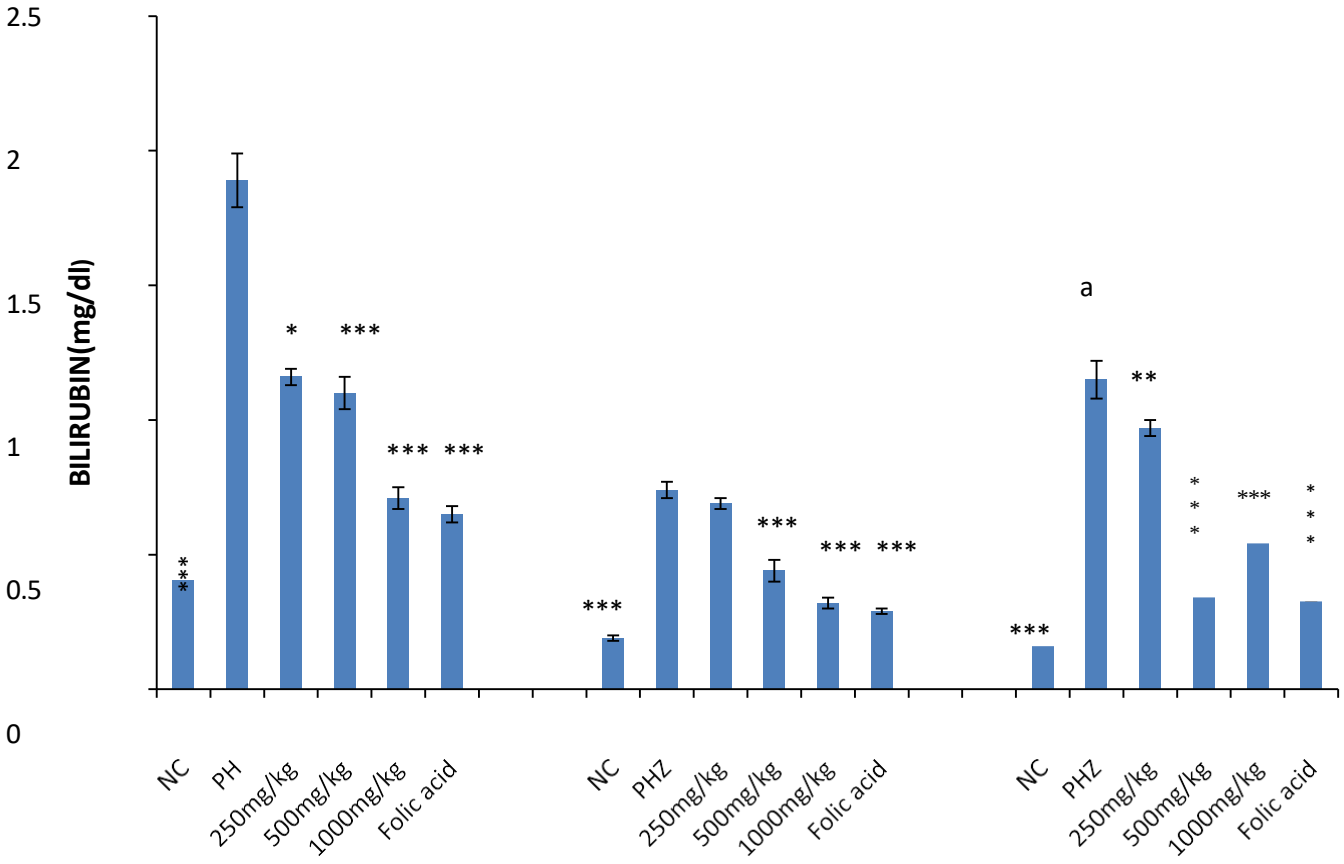


Figure 8: Values represent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's posttest was used to arrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ-treated group while signifies P<0.001 when compared with normal control.

Discussion

The present study evaluated the anti-anaemic activity of *P. niruri* in phenylhydrazine-induced hemolytic anaemia in rats. The results of the study revealed a significant reduction of the weight of rats after induction of anaemia which could be due to the effect of oxidative damages (erythrocytes haemolysis) induced by 2,4-DPH. The reduction in body weight was seen to improve in the group of rats treated with *P. niruri* aqueous extract after 14 days probably due to reversal of the oxidative damage by the extract. This finding is in agreement with some studies reported by [2,4,11]. 2015 where the body weights of rats significantly reduced after induction of anaemia with PHZ but were improved after treatment with 200mg/kg body weight of ethanolic extract of *J. repens* and *C. fascicularis* L and 400mg/kg body weight *Z. jujuba* fruits aqueous extract for 2 weeks respectively.

Berger in 2007 reported that, PHZ injected at a dose of 90 mg/kg body weight to 8 weeks old rats produced a reduction of normal RBC by 45% and PCV by 53% on day 3; reticulocytes by 47% on day 7; and a high MCV of 170% as well as a 60% increase in MCV on day 3. More than 30%, reduction in HB and RBCs was also reported by [4,5,12-16]. In the present study, administration of PHZ in rats produces a decreased PCV level which agreed with previous reports by [17], Maria Claro *et al*., 2006; [5,13] that reported reduction in PCV in hemolytic anaemia induced by 2, 4-DPHZ caused that leads to oxidation of HB and sulfhydryl groups of the erythrocytes membrane and enzymes leading to haemolysis of erythrocytes [17]. However, Oral administration of *P. niruri* whole plant aqueous extract produces a dose-dependent increase in PCV, RBC, HB ($P<0.05$) and a significant ($P<0.05$) decrease in WBC, MCV, MCH and reticulocytes after 14 days treatment. This agreed with earlier findings by [13] where oral administration of 250 and 500mg/kg body weight aqueous extract of *P. erinaceus* Stem Bark significantly increased PCV, Hb, RBC and significantly decreased WBC, MCH and MCHC in PHZ induced anaemic rats probably due to the ability of PNAE to protect the RBC against oxidative haemolysis induced by 2,4-DNPH. The increase in WBC level seen after induction of anaemia might be due to immune stimulatory ability of the chemical similar to what was reported by [18] following administration of ethanolic extract of *P. kurroa* leaves extracts at 100 mg/kg and 200 mg/kg, to PHZ induced anaemic rats which caused increased the level of RBC, PCV and HB in rats. Similarly, treatment of 2,4-DPHZ induced anemic rabbits with *Hibiscus sabdarifa* anthocyanin extract produces a significant ($P<0.05$) increase in, RBC counts, PCV and Hb and a decrease in WBC counts [19].

Conclusion

The current study's findings demonstrated that the whole plant aqueous extract of P. niruri significantly reduced hemolytic anemia in experimental rats caused by 2,4-dinitrophenylhydrazine. The presence of significant phytochemicals including flavonoids and alkaloids, which are known to have erythrocyte-protective properties, may be the cause of the plant's anti-anaemic effect, correcting the rats' anaemia.

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