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Phyllanthusniruri Linn.aqueous extract inhibits 2, 4-dinitrophenylhydrazine-induced anemia in rats.

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Abstract

Jaundice, asthma, hepatitis, the flu, dropsy, diabetes, malaria, hemorrhages, diarrhea, and anemia are just some of the many conditions historically treated using the annual herb Phyllanthusniruri throughout Asia and Africa.The purpose of this research is to determine whether P. niruri has any antianemic properties by analyzing its haemotological and biochemical characteristics in Wister rats with 2,4- dinitrophenylhydrazine-induced haemolytic anemia. This investigation uses haemotological and biochemical parameters and body weight to assess the plant extract's antianaemic activity in Wister rats with 2,4-dinitrophenylhydrazine-induced haemolytic anemia. Folic acid served as a positive control, and three different dosages of plant extract (250, 500, and 1000 mg/ kg b.wt) were given to the test subjects. PCV, Hb, RBC, WBC, MCV, MCH, and Reticulocyte count were among the haematological markers tested. Total and unconjugated bilirubin and oxidative stress indicators (malonyl-CoA dehydrogenase, catalase, and superoxide dismutase) were also measured.

Keywords: *Phyllanthusniruri*; Antianaemic; 2,4-dinitrophenylhydrazine; Haemolyticanaemia; Oxidative stressmarkers

Introduction

One of the most common blood-related illnesses in the world is anemia [1]. Although it may appear at any age, it is more common in pregnant women and small children [2]. Due to the frequency of malaria and other parasite illnesses that may reduce circulating red blood cells or hemoglobin level [3], anemia is more common in the tropics. WHO classifies anemia as a catastrophic public health issue (prevalence > 40%) in 69 countries for children less than five years old and in 68 countries for pregnant women. National Family Health Survey (NFHS- 3) data shows that anemia affects 71% of people in the industrialized world, 84% of those in the developing world, and 79% of people worldwide [4]. Low iron levels are the leading cause of anemia. Malaria, parasite infection, dietary inadequacies, medication toxicity, and inherited or acquired defects are all potential causes of anemia [5].

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Chancapiedra in Spanish, quebrapiedra (stone breaker) in Brazil, and Pitirishi or Budhatri in India are all names for Phyllanthusniruri [6]. In Nigeria, it is known as Iyin-olobe by the Yoruba of the South-West, Egikpeyekpezuma by the Nupes of the North, and Ngwuitekwowanasu by the Igbo of the South-East [7]. Jaundice, asthma, hepatitis, flu, dropsy, diabetes, malaria [9], hemorrhages, diarrheas, dysentery, jaundice, cough, and anemia are only some of the many conditions for which P. niruri has been used in traditional medicine in Asia, Africa, and South America [8]. Bronchitis, anemia, leprosy, asthma, UTIs, stimulating liver, aiding digestion, increasing appetite, and producing laxative effects are only some of the other purported benefits [10]. The Phyllanthusniruri plant has also been employed historically. in the cure of diseases such anemia [7], tuberculosis [8, gonorrhea [9], syphilis [10], and vaginitis [11]. Flavonoids, anthraquinones, alkaloids, saponins, steroids, tannins, and terpenoids were found to be present in P. niruri in a research conducted by Okoli et al. (2010) [8]. Additionally, coumarins and lignans were detected [6]. Extracts of P. niruri (both aqueous and methanolic) have been shown to have significant antioxidant properties in vivo by their capacity to prevent CCl4-induced lipid peroxidation in rats (Harish et al., 2006). As a result, the plant is used in the treatment of a wide range of illnesses, including anemia, all over the world.

Materials and MehodsDrugsand Reagents

2,4-dinitrophenylhydrazine(Britishdrughouse,UK),Folicacid(Emzorpharmaceuticalindustry,Lagos).Otherchemicalsusedwere ofanalytical grade purchased fromBDH,PooleUK.

PlantMaterialandExtraction

Whole, fresh P. niruri plants were gathered in the month of October, 2018, from the city of Minna in the Nigerian state of Niger. A Pharmacognosist from UsmanuDanfodiyo University Sokoto's Department of Ethnomedicine and Pharmacognosy, Faculty of Pharmaceutical Sciences, recognized the plant. A voucher number (PCG/UDUS/Euph/0011) was assigned to a specimen that was submitted to the faculty herbarium.

The plant was shed dried to a consistent weight after a thorough rinsing in water. A pestle and mortar were used to reduce the plant to a powdery consistency. To get 200g/L, we used the cold maceration technique to dissolve 2 kilograms of the powdered plant in 10 liters of distilled water. For three days, the homogenate was stored at 40 degrees Celsius with constant stirring. After straining through muslin, the mixture was filtered again using Whatman No. 1 filter paper. The concentrated filtrate was then kept at 4 C until further use in the research by freeze drying.

Animals GroupingandExperimentalProcedure

Male and female Wister albino rats weighing 180-200g at 12 weeks of age were utilized in the investigation. They were obtained from the Department of Pharmaceutical Sciences at Ahmadu Bello University in Zaria, Nigeria. Prior to the start of the experiment, the animals were housed in standard cages at the animal house of the Faculty of Pharmaceutical Sciences at UsmanuDanfodiyo University Sokoto and given a standard commercial diet from the Vital Feeds company in Jos, Plateau State, Nigeria, as well as free access to water, in order to help them adjust to their new surroundings. Each rat was individually identified with a permanent marker before being randomly assigned to one of many groups using a computer-aided randomization program (Decision analyst stats 2.0). A feeding tube was used to provide the extract to the patient orally. Prior to receiving the extract, each rat was weighed to establish an individual dosage in milligrams per kilogram of body weight. The animals were cared for and used in accordance with the guidelines set out by the Institutional Animal Ethics Committee. The rats' weights were recorded at a variety of time points throughout the study.

InductionofAnaemiausing2,4-dinitrophenylhydrazine

In this investigation, we employed a variant of the technique described by Berger (2007). The duration of the trial was 30 days. There were a total of eight animals used to create the five categories. Once a day through feeding canula, all the animals were given 2,4-DNPH (20 mg/kg b.wt, p.o.) for 7 days. On day 8, hematological samples were taken from the rats by puncturing their sinuses in the tail veins and collecting the blood in heparinized capillary tubes. Rats with a PCV drop of 30% or more were declared anemic and utilized in the experiment [5].

PCV was measured before and after inducing anemia by collecting blood from a small incision near the tail and letting it flow through a capillary tube until it filled more than two-thirds of the tube. A Bunsen burner was used to smolder the tube's end off. For 5 minutes at 3,000 rpm, the samples were centrifuged. The PCV was measured by placing capillary tubes on the microhaematocrit.

As soon as anemia was diagnosed, treatment with the

extract was initiated. Plant extracts of 250, 500, and 1000mg/kg were administered to the treatment groups, whereas the positive control group received 75 micrograms per kilogram of folic acid and the negative control group received no treatment. The oral feeding cannula was used once a day for 14 days straight to provide all therapies.

HaematologicalAssay

Blood samples were taken through cardiac puncture and stored in EDTA vials to avoid clotting after the aqueous extract had been administered for two weeks. Haematological parameters (including RBC, PCV, Hb, WBC, MCHC, MCV, and reticulocyte count) were calculated from the samples. Except for reticulocytes, all haematological parameters were measured automatically using a Sysmex Machine (Model No KX-21N) from Sysmex Laboratories Ltd in the United States. The blood samples were centrifuged or blended before being run through an Automated Analyzer, a device that determines the total quantity and composition of the blood's various cell types. The number of reticulocytes was counted visually [20].

Lipidperoxidationassay(Thiobarbituricacidmethod)Principle

MDAreactwiththiobarbituric acid(TBA)toformanMDA-TBA2adductthatabsorbsstronglyat532nm.

Procedure

As described by (David et al., 1990) 150µL of the sample was diluted to 500µL with double deionized water. In testtubes,250µLof1.34%Thiobarbituricacidwasadd edfollowedbytheadditionofequalvolumeof40%tric hloroaceticacid.Themixturewaswellshakenandincu batedfor30minutesinboilingwater(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 wascalculated usingthe followingformula.

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

Theprincipleofthismethodisbasedonthecompetitionbetw eenthepyrogallolautoxidationbyO2•¯andthedismutation ofthisradical bySOD.

Procedure

Test tubes were labeled as the test and blank and 0.1mL of Buffer was added into test labeled tubes while 0.15mLwas 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^{0}C for 10 minutes then transferred into cuvette. 0.02mL ofPyrogallolwas added to both blank and test. Themixture is immediately mixed by inversion and changeinabsorbance at 240nm was recorded after 3 minutes approximately. The change in absorbance at 420nm/minute iscalculated usingthemaximumlinearrate for bothtestandblank.

$$\%\text{inhibition}=\frac{\text{Absat420nm/minofblank}-}{\text{Absat420nm/minof samplex100}}$$

Abs420nm/minofblank

Abs–Absorbance

ThenumberofunitsofSODiscalculatedusingthe followingformula: %inhibition/(100-%inhibition)=Units/ml

OneunitofSODactivitywasdefinedastheamountofe nzymethatreducestherateofauto-oxidationofpyrogallolby50%atstandardassaycondit ions.

BilirubinPrinciple

Bilirubin reactswith diazotized Sulphanilicacid to form a colored azobilirubin compound. The unconjugatedbilirubincoupledwiththeSulphanilica cidindpresenceofcaffeineproducesacolourcomplex also.Theintensityofthecolorisdirectlyproportionalt o theconcentrationofBilirubininthespecimenand ismeasured at546 nm.

Total BilirubinProcedure

As described by (Olukunle*et al.*,2010). Test tubes

were labeled as blank and sample 200µL of reagent 1 was addedto 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 bothsample and blank tubes. The mixtures were incubated at 20-25^{0}C for 10 minutes followed by the addition of 1000µLofreagent.Thecontentofthetubesweremixed andincubatedat25^{o}Cfor5-30minutes.Thecontentofthetubeswas transferred into a Cuvette and absorbance was recorded at 546nm. The concentration of total Bilirubin wascalculated usingthe followingformula.

Totalbilirubin(µm/L)=185
XAbsorbanceoftotalbilirubin(546nm)

DirectBilirubin

Test tubes were labeled as blank and sample, 200µL of reagent 1 waspippetted into both blank and sample testtubes. Followed by the addition of 50µL of reagent 2 into sample test tubes. Addition of 0.9% NaCl (200µL) wasdone to both sample and blank test tubes followed by the addition of 200µL of the test sample (serum) into bothsample and blank test tubes. The content of the tubes was mixed and incubated for 10min at 20-25^{0}C. Absorbancewastakenat546nm.The concentrationofdirectBilirubinwascalculatedusingthefollowingformula.

Directbilirubin(umo/L)=246
XAbsorbanceofdirectbilirubin(546nm)

StatisticalAnalysis

The Datawere expressedasmean ±standarderror andwere analyzedusing One-wayAnalysis of Variance(ANOVA).Thecomparisonbetweenuntreatedand Treatmentgroupswasdoneusingthe Dunnet's multiplecomparisontests. The level of significancewassetat $P < 0.05$.

Results

Following the induction of anaemia, body weights of the rats pre and post induction of anaemia with PHZ wasevaluated. 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 comparedwiththe untreatedgroupasseeninFigure1below.

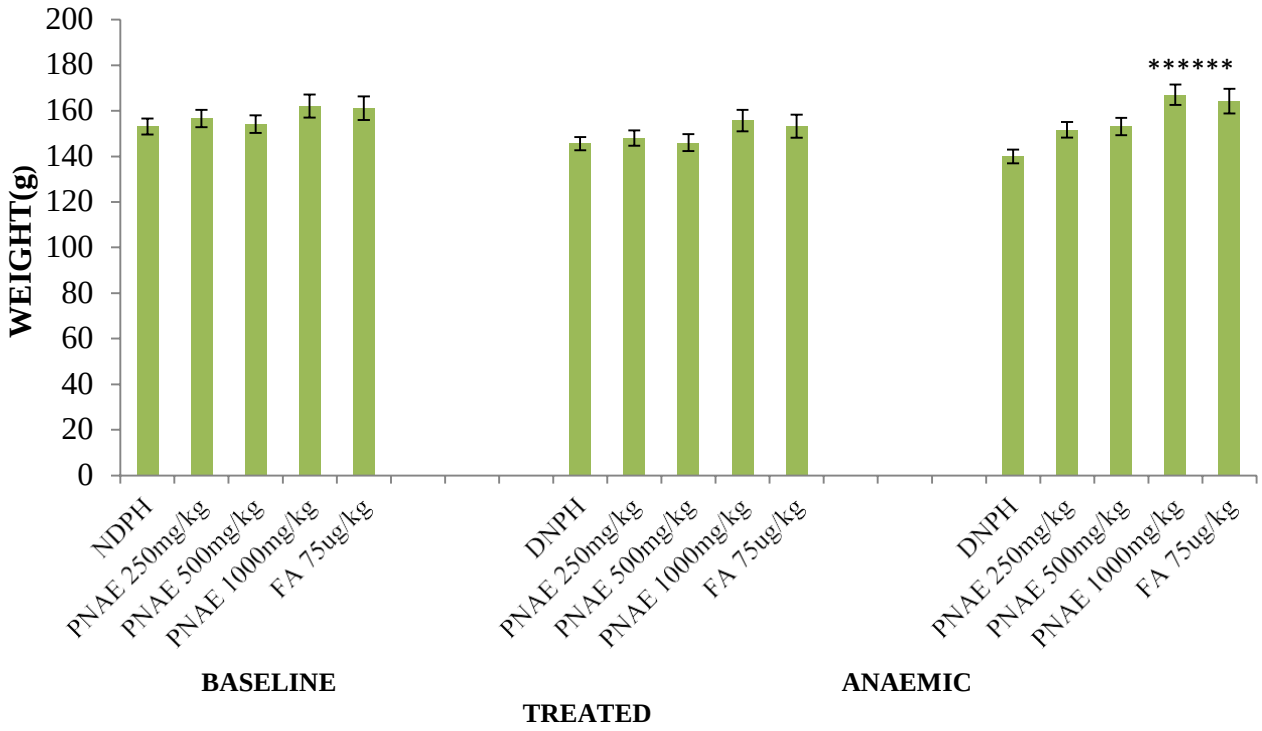

Figure 1: Effect of *P. niruri*Aqueous Extract on weight of Rats pre and post induction of anaemia 14 days aftertreatment with PNAE. Values represent the means ± SE (n= 8 rats each for all the groups). P<0.05 and P<0.001 wasconsidered significant when compared with untreated group. PNAE = *P niruri*aqueous 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 PHZtreated 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 asillustrated inFigure2below.

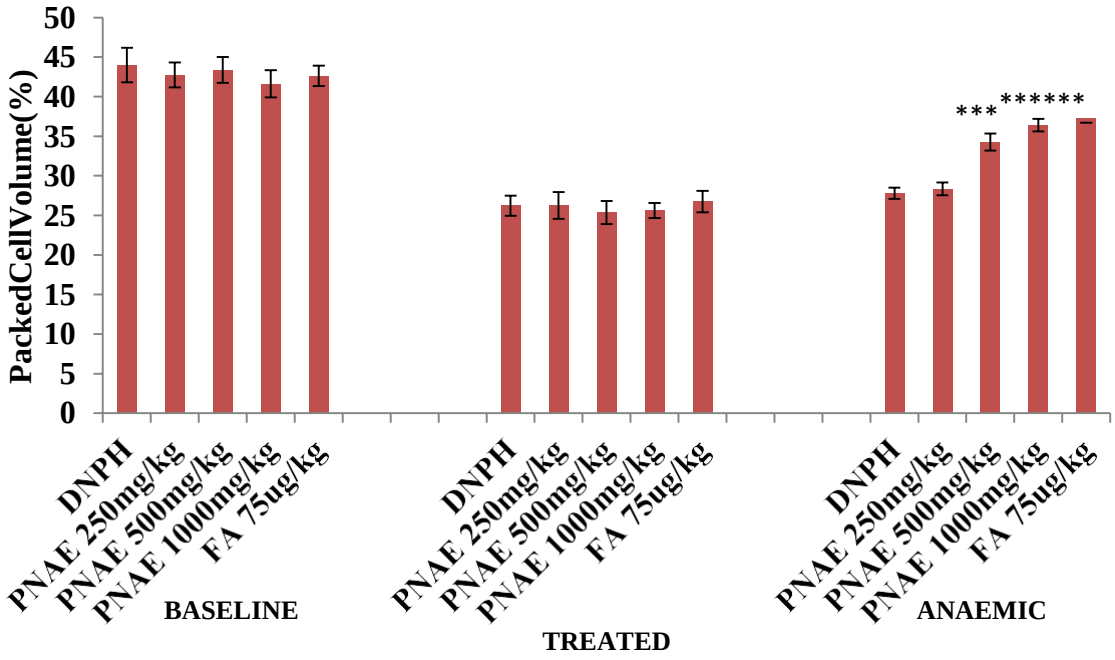

Figure 2: Effect of *P. niruri*aqueous extract on PCV in 2,4-DPHZ-induced anaemic rats at baseline, after inductionof anaemia and after 14 days treatment with PNAE. Values represent the means ± SE (n= 8 rats each for all thegroups). One-way ANOVA with Dunnet's posttest was used to arrive at the P values.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

The treatment of PHZ induced anemic rats with PNAEfor 14 days shows a significant (P>0.001) anddose-dependent increase in Hb concentration and also a significant (P<0.001) decrease in the untreated control group asillustrated inthe Figure3below.

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 comparedwith the untreatedgroup.There was also asignificant (P<0.001) decrease when PHZ group was compared to the control after treatment as shown in Table 1below.

TheresultofWBCconcentrationfollowingtreatmentofPHZinducedanemicratswithPNAEProducesasignificant (P<0.02) and dose-dependent decrease in WBCswhen compared with the untreated group. There wasalso a significant (P<0.001) increase in WBC level when PHZ group was compared to the control after treatment asshown inTable 1below.

Table1: Effectoftreatmentofanemicratswithaqueousextractof*P.niruri*onHB,RBCs,andWBCs

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*
Folicacid (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 whencompared tountreatedgroups.

Also, a significant (P<0.05) decrease in MCV, MCH and reticulocytes was observed at 500 and 1000mg/kg after 14days treatment with 500 and 1000mg/kg body weight when compared to the 2,4-DNPH induced anemic group of theextract.Nosignificant(P>0.05)effect wasobservedatadoseof250mg/kgbodyweightasshowninTable2below.

Table2: Effectoftreatmentofanemicrats withaqueousextractof*P.niruri*onMCV,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*
Folicacid(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 whencompared tountreatedgroup.

Biochemical ParametersOxidative Stress MarkersMalondialdehyde

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). Therewas also a significant ($P<0.001$) increase in MDA levelwhen PHZ group was compared to the control aftertreatmentasshownin Figure3below.

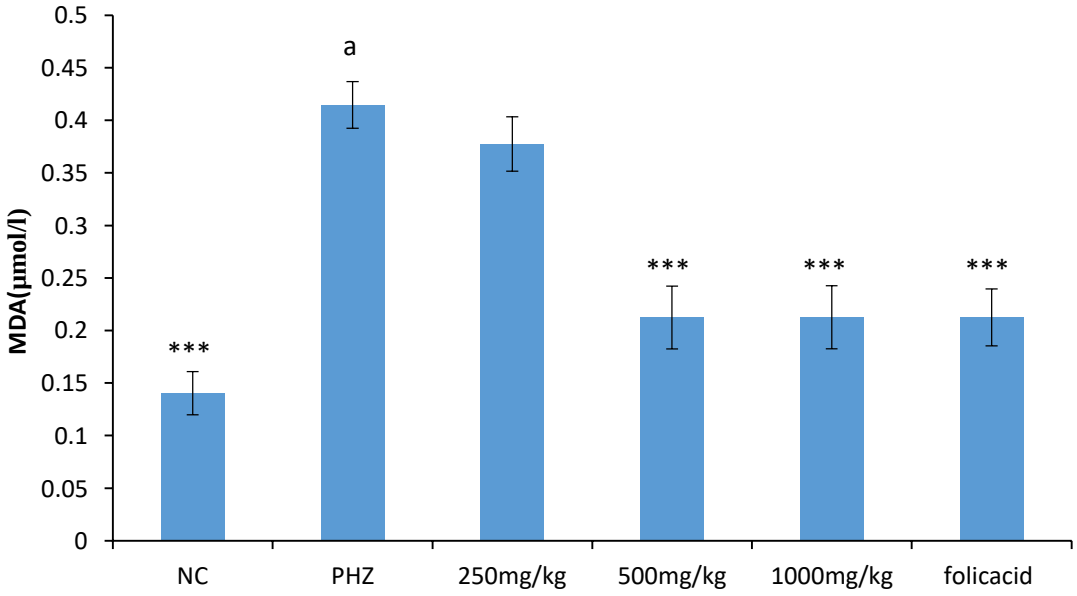

Figure 3: Effect of PNAE on MDA in PHZ-induced Anaemic Rats after 14 days treatment with PNAE. Valuesrepresent the means ± SD (n= 8 rats each for all the groups). Oneway ANOVA with Dunnet's post-test was used toarrive at the P values. *, ** and *** signifies $P<0.005$, $P<0.02$ and $P<0.001$ respectively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

SuperoxideDismutaseStimulatoryActivity ofPNAE

TheresultofSODactivityfollowingtreatmentof PHZinducedanemicratswithPNAEshowsasignificant($P<0.01$) and dose-dependent increase in SOD activity when compared with the untreated group (PHZ). There wasalso a significant ($P<0.001$) decrease in SOD activity level when PHZ group was compared to the control aftertreatmentasshowninFigure4below.

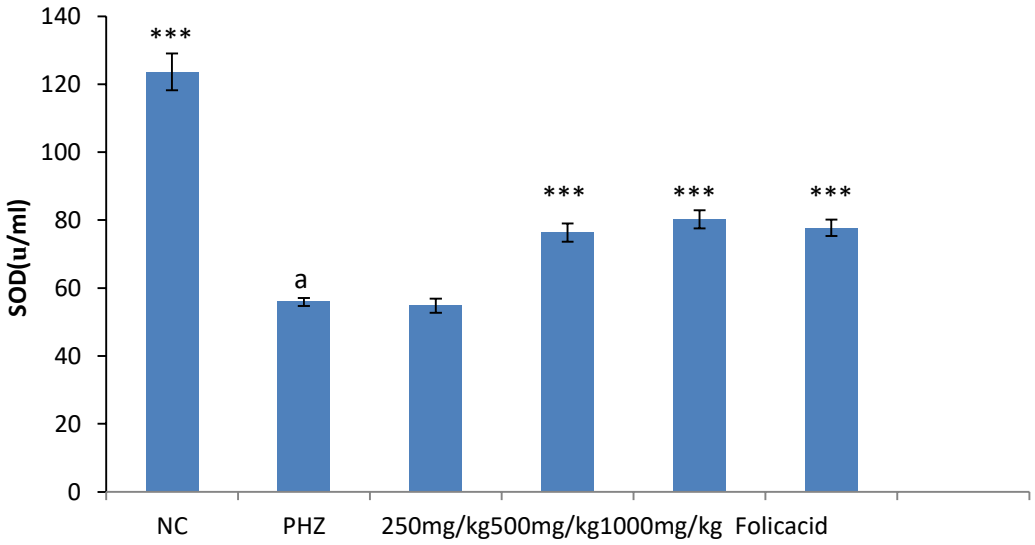

Figure 4: Effect of PNAE on SOD in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Valuesrepresent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post- test was used toarrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively when compared with PHZ-treated groupwhile ᵃsignifiesP<0.001whencomparedwithnormalcontrol.

Effectoftreatmentoncatalaseactivity

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). Therewas also a significant (P<0.001) decrease in catalase activity when PHZ group was compared to the control aftertreatmentasshowninFigure5below.

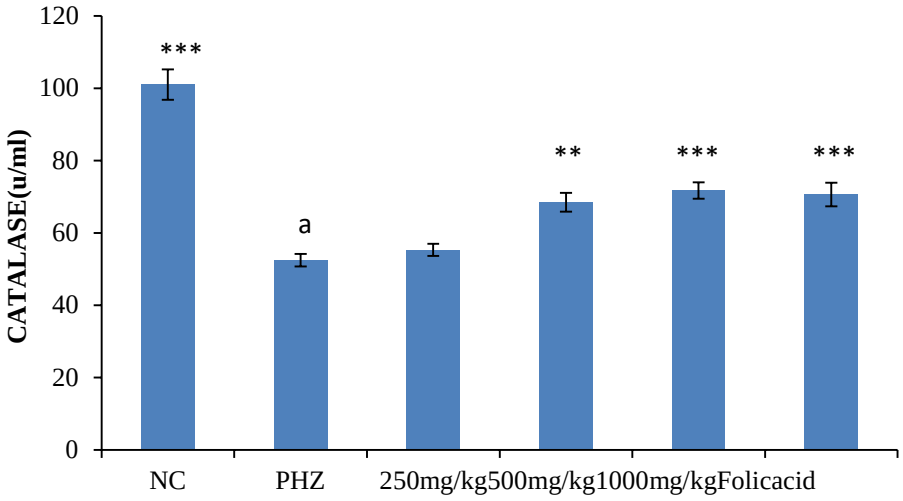

Figure 5: Effect of PNAE on Catalase in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Valuesrepresent themeans±SD(n=8rats eachforallthegroups).One-wayANOVAwith Dunnet's post-testwasusedto

arriveatthePvalues.*,** and***signifiesP<0.005,P<0.02andP<0.001respectivelywhen comparedwith PHZ-treated group.while[a]signifies p<0.001whencomparedwithnormalcontrol.

Biochemical ParametersOxidative Stress MarkersMalondialdehyde

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). Therewas also a significant (P<0.001) increase in MDA levelwhen PHZ group was compared to the control aftertreatmentasshownin Figure6below.

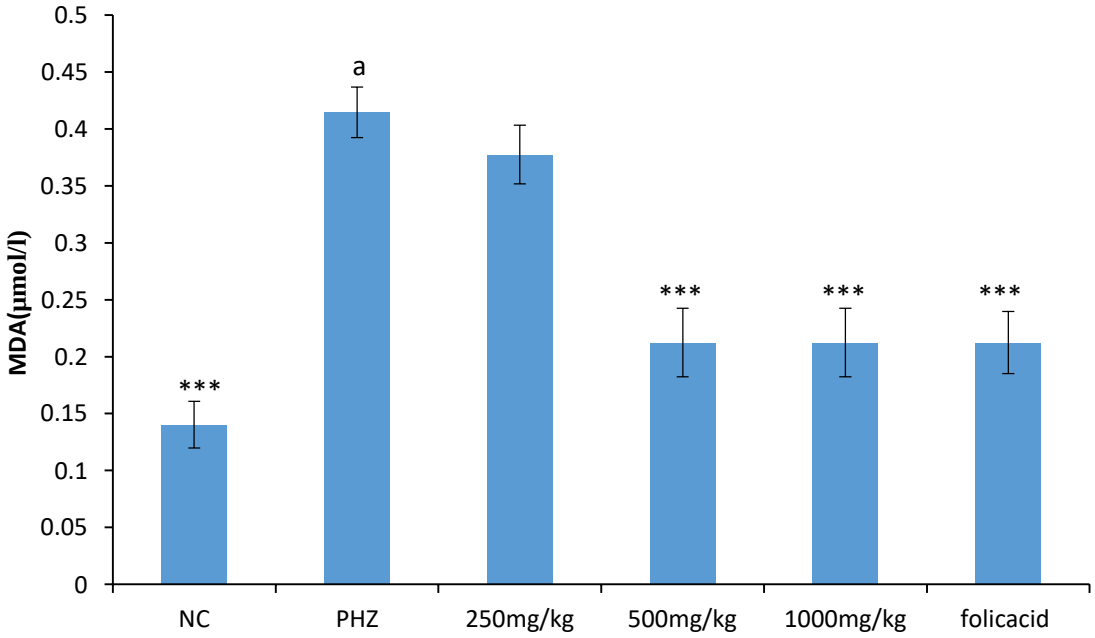

Figure 6: Effect of PNAE on MDA in PHZ-induced Anaemic Rats after 14 days treatment with PNAE. Valuesrepresent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post-test was used toarrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively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

SuperoxideDismutaseStimulatoryActivity ofPNAE

TheresultofSODactivityfollowingtreatmentofPHZinducedanemicratswithPNAEshowsasignificant(P<0.01) and dose-dependent increase in SOD activity when compared with the untreated group (PHZ). There wasalso a significant (P<0.001) decrease in SOD activity level when PHZ group was compared to the control aftertreatmentasshownin Figure7below.

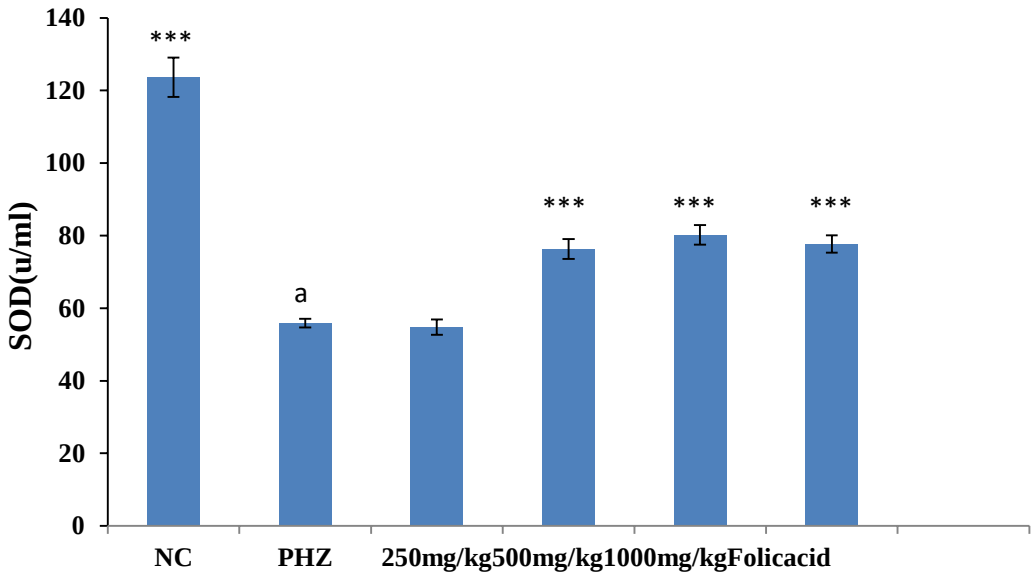

Figure 7: Effect of PNAE on SOD in PHZ-induced Anaemic Rats after 14 days of treatment with PNAE. Valuesrepresent the means ± SD (n= 8 rats each for all the groups). One-way ANOVA with Dunnet's post-test was used toarrive at the P values. *, ** and *** signifies P<0.005, P<0.02 and P<0.001 respectively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

Effectof TreatmentonBilirubin

The result of bilirubin assay revealed a significant (P<0.001) and dose-dependent reduction in total, direct andunconjugated bilirubin when PHZ inducedanaemic rats were treated with PNAE compared with the untreatedgroup. There was also a significant (P<0.001) increase in bilirubin when PHZ group was compared to the controlafter treatmentasshowninFigure8below.

Discussion

This investigation tested P. niruri for its ability to reverse phenylhydrazine-induced hemolytic anemia in rats. The research found that 2,4-DPH-induced anemia led to a considerable decrease in the weight of rats, which may have been the consequence of oxidative damages (erythrocytes haemolysis). After 14 days, rats administered with P. niruri aqueous extract showed improved body weight loss, likely owing to the extract's ability to reverse oxidative damage. Some research, such as those cited in [2,4,11], corroborate this conclusion. 2015 study in which rats treated with 200mg/kg body weight of ethanolic extract of J. repens and C. fascicularis L and 400mg/kg body weight of Z. jujuba fruits aqueous extract for 2 weeks showed better body weights following PHZ-induced anemia.

Injecting 90 mg/kg of body weight of PHZ into 8-week-old rats, as reported by Berger in 2007, decreased normal RBC by 45% and PCV by 53% on day 3; reticulocytes by 47% on day 7; and increased MCV by 170% and MCV by 60% on day 3. The percentage of HB and RBCs has decreased by more than 30%, as reported by [4,5,12-16]. Consistent with previous reports [17], Maria Claro et al., 2006; [5,13] found a reduction in PCV in hemolytic anemia induced by 2, 4-DPHZ, which causes oxidation of HB and sulfhydryl groups of the erythrocytes membrane and enzymes leading to haemolysis of erythrocytes, when given to rats in the present study. After 14 days of therapy, however, peripheral blood cell volume (PCV), red blood cell hemoglobin (HB), and white blood cell (WBC) count are all significantly (P0.05) increased whereas WBC count, MCV, MCH, and reticulocyte count are all significantly (P0.05) decreased. The ability of PNAE to protect the RBC against oxidative haemolysis induced by 2,4-DNPH was confirmed in PHZ-induced anemic rats when an aqueous extract of P. erinaceus Stem Bark was orally administered at 250 and 500mg/kg body weight, as previously reported by [13]. Similar to what was reported by [18] after administering ethanolic extract of P. kurroa leaves extracts at 100 mg/kg and 200 mg/kg, to PHZ induced anemic rats, which caused increased level of RBC, PCV, and HB in rats, the increase in WBC level seen after induction of anaemia may be due to the immune stimulatory ability of the chemical. Similar increases (P0.05) in RBC counts, PCV, and Hb, as well as decreases (P0.05) in WBC counts, are seen in 2,4-DPHZ-induced anemic rabbits treated with Hibiscus sabdarifa anthocyanin extract [19].

Conclusion

The current investigation found that 2,4-dinitrophenylhydrazine-induced haemolytic anemia in experimental rats responded well to a treatment with a whole plant aqueous extract of P. niruri. The plant's ability to reverse the anemia caused in rats may be attributed to the presence of key phytochemicals such flavonoids and alkaloids, which are known to possess erythrocytes protecting qualities.

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