

Elemental compositions and anti-anemic property of *Harungana madagascariensis* stem bark

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Abstract

The concentrations of 15 elements and heavy metals in the stem bark of *Harungana madagascariensis* were determined using an energy-dispersive X-ray fluorescence (EDXRF) spectrometer. The anti-anemic activity was done using the changes in hematological parameters (PCV, RBC and Hb) influenced by phenylhydrazine HCL (80 mg/kg) and malaria parasites-induced anemia. Results shows Cd, Ni, Mo, Cr and Br were in the range of 0.021–0.94 mg/g, while Pb, Zn, Fe, Cu and Hg were in the range of 1.50–7.24 mg/g. The elements with very high concentration were Ca, K, Sr, Mn, and Cl and were in the range of 10.5–774.3 mg/g. Remarkable anti-anemic activity was obtained with PCV of 40–48%, RBC count of $81-155 \times 10^4$ and Hb value of 57–66 g/dL after treatment compared with 30% PCV, 67×10^4 RBC count and 36.5 g/dL Hb value obtained for the untreated control animals. Our results suggest that *H. madagascariensis* stem bark extract constituents' exhibit anti-anemic activity.

Introduction

Plasmodium falciparum malaria remains a massive burden of disease with an estimated two million deaths worldwide each year and responsible for two major complications, namely; cerebral malaria and severe anemia (Bremner, 2001). Anemia is a major cause of morbidity and mortality in malaria endemic areas of tropical regions of the world. Anemia in malaria is caused by destruction of red blood cells in the body or the depression of red blood cell production in the bone marrow. As the red blood cells become less, the patient weakens. In severe cases the patient is unable to deal with basic life essential tasks and may die. There are various causes and types of anemia, these include: sickle-cell anemia, iron deficiency anemia, vitamin B₁₂ anemia, drug induced anemia as side effect of drug therapy.

In the treatment of diseases like malaria and anemia, the beneficial effects of analyzed drug materials especially from plants are mainly attributed to the

presence of constituents (alkaloids, saponin, terpenoids, anthraquinones, essential oils, flavonoids, tannins, etc). The efficacy of these constituents could also be influenced by inorganic components known as the macro and micro (trace) elements. There are basically 17 important elements known to be required by all higher plants. Nine of them are macronutrients (C, N, O, H, K, Ca, Mg, P and S). An additional eight elements are defined as micronutrients (B, Cl, Cu, Fe, Mn, Mo, Ni and Zn). These micronutrients (Fe, Mn and Zn) are important as co-factors for enzymes activities, Cu is bound to amino acids, while some elements, such as Mn, Nd and Ce, are bound to some bio-macromolecules forming coordination compounds. Fe, Cu, Mn, Co, are important components of many antioxidant processes in human body, a deficiency of any of these essential elements may impair the function of the overall oxidant mechanisms in both plants and animals. Therefore, accurate quantitative analysis of the elemental content of plants is very important, as a contribution to increasing concern about their potential effects on



human health (Slavica et al., 2005). To mention just few examples, the following medicinal plants have been employed in the management of anemia: The stems with leaves of *Indigofera tinctoria*, barks stem, branch, trunk of *Khaya senegalensis*, leaves of *Sorghum vulgare*, roots of *Uvaria chamae*, decoction were used in West Africa. Because of the prominent iron contents of 35.69 and 35.21 mg/100 g found in the root bark of *Bridelia cathartica* and *Lannea stuhlmannii*, respectively, they were useful in East Africa for anemia. Also, in China and India, *Rehmannia glutinosa* and *Boerhavia diffusa* were used for anemia (Gupta and Nandyala, 1984; Duke and Ayensu, 1985; Adjanohoun, et al 1986; Omolo et al., 1997). This study was carried out to determine the elemental compositions and examine the efficacious claim of the use of juice and sap from the *Harungana madagascariensis* stem bark in the management of iron deficiency anemia induced in pregnancy and child birth in folklore medicine. The study was done using two main modes of anemic induction through malaria and phenylhydrazine drug induced anemia.

Materials and Methods

Drugs and chemicals: Phenylhydrazine hydrochloride (Sigma), artemether (Sigma), vitamin B₁₂ (Jiano Bu Pharmaceuticals, China), 95% ethanol (BDH) were used.

Plant materials and preparation of the extracts: *H. madagascariensis* stem bark samples were collected near the main University campus, Ile-Ife. The plant was identified by Mr. O. A. Oladele, Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife and a voucher specimen (No. FHI 107392) was kept at the herbarium of the Forestry Research Institute of Nigeria, Ibadan. The ethanol extraction was made by soaking 325 g of the powdered stem bark in 70:30 ethanol:water for 24 hours. After maceration, the extract was evaporated *in vacuo* in a rotary evaporator to dryness, yielding 9g of the brown crude extract.

Elemental compositions analysis: The elemental analysis of the plant material was performed by an energy-dispersive X-ray fluorescence (EDXRF) spectrometer. The spectrometer consisted of a Siemens FKO-04 tube with Mo anode, a kristalloflex 710H X-ray Generator installed in the Centre for Energy Research and Development, Obafemi Awolowo University, Ile-Ife. The equipment is operated under QXAS (Quantitative X-ray Analysis System) software (QXAS, Manual 1993). The sample and standard formulated pellet was irradiated for 20 min in a fixed tube at 30 kV and 10 mA. The samples was analyzed before the standard. All

the values obtained are results of an average of three measurements on the plant material.

Animals: Mice and rats of either sex, weighing between 18-22 g and 180-250 g respectively were used. The animals were maintained at 25 ±1°C under natural 12 hours daylight/night conditions for at least 5 days before the experiment. All the animals were fed with standard diet in the Department of Pharmacology Animal House and water was given *ad libitum*. The "principle of laboratory animal care" (National Institute of Health- NIH publication No. 85-23) guidelines and procedures were followed in this study (NIH publication revised, 1985).

Parasites: The N67 chloroquine sensitive strain of *Plasmodium yoelii nigeriensis* was donated generously by the Malaria Research and Reference Reagent Resource Center (MR4) Manassas, VA, USA, and was obtained from Institute of Medical Research and Training courtesy of Dr. O. G. Ademowo.

Anti-anemia activity: Anti-anemic activity was assessed using a) drug induced anemia using phenylhydrazine hydrochloride and b) malaria-induced anemia. The animals were randomly distributed into the following six groups: Group 1: non-anemic control without treatment, Group 2: anemic control without treatment, Group 3: anemic rats treated with vitamin B₁₂, Group 4: anemic rats treated with 20 mg/kg extract, Group 5: anemic rats treated with 40 mg/kg extract, Group 6: anemic rats treated with 80 mg/kg extract

The experiment in drug-induced anemia using phenylhydrazine was performed for 3 weeks. All the rats (except the ones in the first group) were injected with a single subcutaneous dose of 80 mg/kg of phenyl hydrazine hydrochloride (Yeshoda 1942; Berger 1985). On day 0, before initiation of anemia, initial weight, PCV, RBC and Hb were taken in all the groups. Anemia was allowed to establish in 24 hours (Nath and Prasannan, 1958). On the day of establishment of anemia, the weight, PCV, RBC and Hb were taken in all the groups. Treatment with the extract (20, 40, 80 mg/kg), vitamin B₁₂ (100 µg/kg), commenced 24 hours after the administration of phenylhydrazine hydrochloride and continues for 21 days on daily basis. Measurement of PCV, RBC and Hb were taken daily for the first 3 days then on day 5, 7, 9, 13, 17 and 21.

For the malarial-induced anemia experiment, six groups of mice were inoculated with a standard inoculum size of 1 × 10⁷ (0.2 mL) infected erythrocytes of *P. yoelii nigeriensis* intraperitoneally on day zero. Using the 4 day schizonticidal suppressive mode of treatment, the extracts (20, 40, 80 mg/kg), vitamin B₁₂ (100 µg/kg), artemether (25 mg/kg) were given to the mice for 4 days. Measurement of PCV, RBC and Hb were taken at day 0, 3 and 6.

Packed cell volume determination: PCV of the rats was determined by obtaining blood from a slightly cut rat tail and allowing the blood to flow through a capillary tube to more than two-third of the tube. The end with blood was then sealed off using plasticine. The capillary tubes were placed in the centrifuge and centrifuged for 15 min at 3,000 rpm. The capillary tubes were placed on the microhemotocrit to determine the packed cell volume.

Red blood cell count determination: Using the red blood cell pipette, the rat's blood is sucked to 0.5 mL mark. The mouth of the pipette was wiped using a tissue paper and hayem solution was sucked to 101 mark. The blood and hayem solution were properly mixed. The mixture is placed in between the cover slide and the counting chamber of the hemocytometer. The red blood cells were counted under the microscope.

Hemoglobin level determination: The dilution tube was filled to the mark 10 with freshly prepared 0.1N HCL. Blood was sucked to 20 mL mark of the pipette from a slightly cut tail of a rat and blown gently into the acid. It was allowed to stand for 5 min for the acid hematin to form. Distilled water was added drop by drop and stirred with a rod after each addition. This was continued until the dilution tube is slightly darker than that of the standard at daylight against a white background. More distilled water was added until a slightly lighter color was obtained. The average of the value obtained for slightly darker and slightly lighter was taken as hemoglobin level.

Statistical analysis: Data are expressed as mean \pm S.E.M and analyzed using Students' t test. The significant level was set at $p < 0.05$. The IC_{50} values were calculated using the Microsoft Excel program.

Elements	Concentration (mg/g)	Comment
Bromine	0.94	Very low concentration
Cadmium	0.05	Very low concentration
Calcium	774.00	Very high concentration
Chloride	10.50	High concentration
Chromium	0.08	Very high concentration
Copper	1.53	Moderately low concentration
Iron	7.24	Moderately low concentration
Lead	2.11	Moderately low concentration
Manganese	33.40	Moderately low concentration
Mercury	1.50	Moderately low concentration
Molybdenum	0.02	Very low concentration
Nickel	0.07	Very low concentration
Potassium	164.00	Very high concentration
Strontium	11.20	High concentration
Zinc	4.55	Moderately low concentration

Results

The elemental analysis of *H. madagascariensis* stem bark sample revealed the presence of fifteen elements and heavy metals, Ca, K, Mn, Sr, Cl, Br, Cr, Cd, Cu, Fe, Ni, Pb, Zn, Mo and Hg in three categories of various concentrations. For example, Cd, Ni, Mo, Cr, Br were in the range of 0.021–0.94 mg/g, while Pb, Zn, Fe, Cu and Hg were in the range of 1.50–7.24 mg/g. The elements with very high concentration were Ca, K, Sr, Mn, and Cl and were in the range of 10.5–774.3 mg/g (Table I).

Table II showed the effects of the extract on the anemia induced by malaria parasites on selected hematological parameters of albino mice. Administrations of the extract to the animals at various doses (20, 40 and 80 mg/kg body weight) produced significant increase ($p < 0.05$) in the hemoglobin content compared to the control mice; however, the values are not in dose dependent manner. Remarkable anti-anemic activity was obtained with PCV of 40–48%, RBC count of $81\text{--}55 \times 10^4$ and Hb value of 57–66 g/dL after treatment compared with 30% PCV, 67×10^4 RBC count and 36.5 g/dL Hb value obtained for the untreated animals.

Table II: Anti-anemic property of *H. madagascariensis* stem bark extract against *Plasmodium yoelii* nigeriensis (rodent malaria parasite) induced anemia

Treatments (mg/kg) / days	PCV (%)			RBC			Hemoglobin (g/dL)		
	D0	D3	D6	D0	D3	D6	D0	D3	D6
Control	54.3	41.3	30.0	130×10^4	99×10^4	67×10^4	81.3	55.7	36.5
Extract 20	53.0	45.7	48.0	164×10^4	110×10^4	155×10^4	76.7	56.7	60.0
Extract 40	45.3	32.0	40.0	140×10^4	119×10^4	105×10^4	58.0	43.0	66.0
Extract 80	59.0	47.0	47.0	142×10^4	111×10^4	81×10^4	90.3	66.7	65.0
Vitamin B ₁₂ (100 μ g/kg)	47.7	36.3	39.0	139×10^4	86×10^4	83×10^4	79.0	56.0	57.0
Artemether (25 mg)	57.3	46.7	34.7	161×10^4	94×10^4	125×10^4	79.3	75.7	57.3

According to Dhawan and Srimal (1997), the normal values for PCV, RBC count and Hb in normal control rats are 40–52%, $6.5\text{--}9.0 \times 10^4$ and 60.4–76.8 g/dL respectively

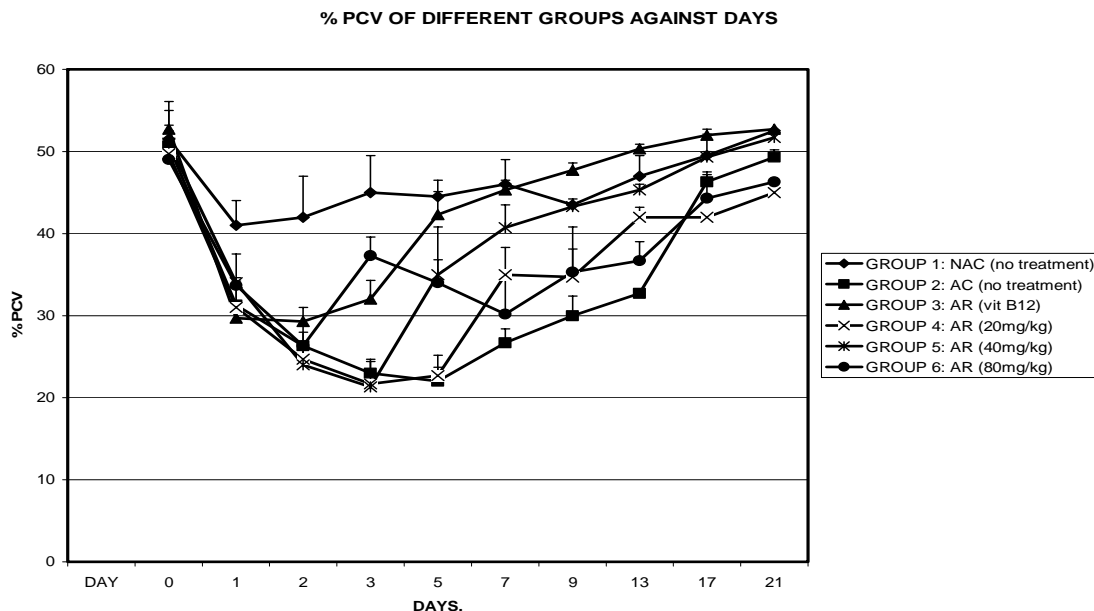


Figure 1: Anti-anemic effect of *Harungana madagascariensis* stem bark extract on PCV (packed cell volume) of rats with anemia induced by phenylhydrazine (80 mg/kg). Group 1: Non-anemic control without treatment; Group 2: Anemic rats without treatment; Group 3: Anemic rats treated with vitamin B₁₂; Group 4: Anemic rats treated with 20 mg/kg extract; Group 5: Anemic rats treated with 40 mg/kg extract; Group 6: Anemic rats treated with 80 mg/kg extract

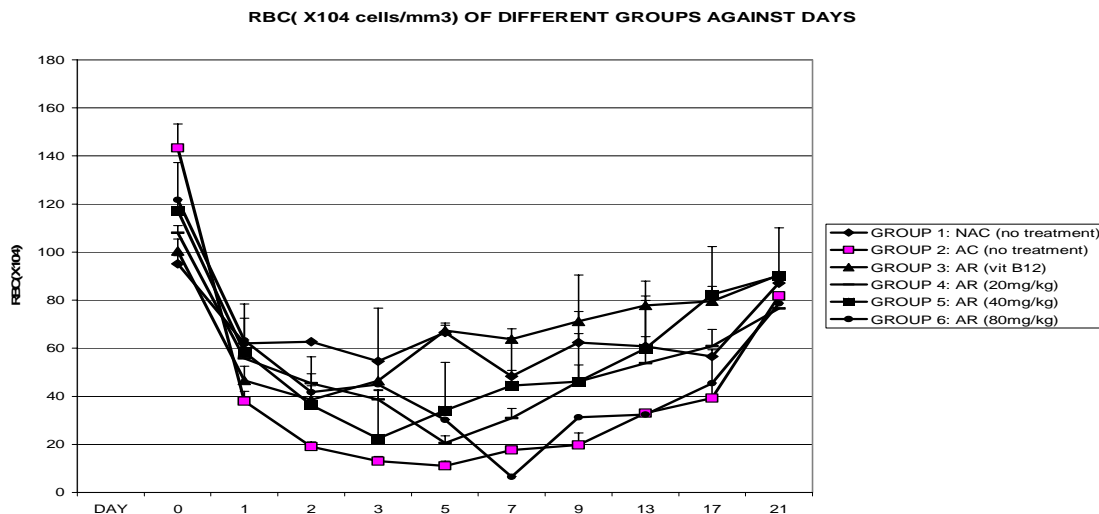


Figure 2: Anti-anemic effect of *Harungana madagascariensis* stem bark extract on RBC (red blood cell) of rats with anemia induced by phenylhydrazine (80 mg/kg). Group 1: Non anemic control without treatment; Group 2: Anemic rats without treatment; Group 3: Anemic rats treated with vitamin B₁₂; Group 4: Anemic rats treated with 20 mg/kg extract; Group 5: Anemic rats treated with 40 mg/kg extract; Group 6: Anemic rats treated with 80 mg/kg extract

Figures 1, 2 and 3, showed the effects of the extract on the anemia induced by phenylhydrazine hydrochloride in rats. The three selected hematological parameters (PCV, RBC and Hb) significant decreased ($p < 0.05$). However, vitamin B₁₂ and the extract increased significantly ($p < 0.05$) these hemoglobin contents (Figure 1, 2, 3). This similar pattern of increase in PCV, Hb and RBC was also observed from day 5 to day 21. These increases were significant ($p < 0.05$), however not dose dependent.

Discussion

Practical approach to malaria is essential if the global objectives of malaria control are to be achieved. Anemia is a universal feature of malaria. It is common among the general population in the tropics, particularly in Africa and especially in children and pregnant women (Beales, 1997). In plants and animals development, their metabolic reactions require some essential elements. These elements are also important for diseases and disorders control in man. However, they could produce

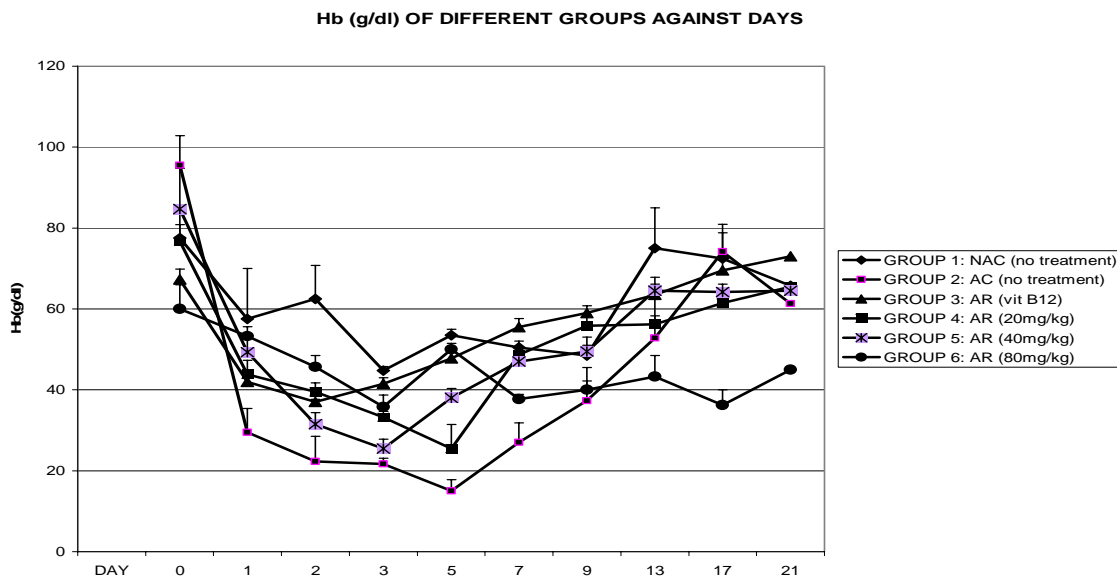


Figure 3: Anti-anemic effect of *Harungana madagascariensis* stem bark extract on Hemoglobin (Hb) contents of rats with anemia induced by phenylhydrazine (80 mg/kg). Group 1: Non-anemic control without treatment; Group 2: Anemic rats without treatment; Group 3: Anemic rats treated with vitamin B12; Group 4: Anemic rats treated with 20 mg/kg extract; Group 5: Anemic rats treated with 40 mg/kg extract; Group 6: Anemic rats treated with 80 mg/kg extract

harmful effects in excessive concentrations; therefore, their different roles in the physiological processes strongly depend on their concentrations in a particular plant tissue. The plant analyzed in this work is known all over the world for their different pharmacological activities and, thus, have a very important place in phytotherapy (EMEA 1999; Gurib-Fakim 2007). Toxic heavy metals such as Cd, Pb, and Hg were detected in the sample of *H. madagascariensis* stem bark, but they were of minimal concentrations. Trace elements (e.g., B, Cu, Mn, Ni, Zn) that are phytotoxic at concentrations not harmful to human are of much less concern than elements that are easily concentrated in plants at toxic levels to humans and animals (e.g., Cd, Co, Se, Mo). According to the EU Commission Regulation published by Kabata-Pendias and Mukherjee (2007), maximum amount of Cd, Pb, and Hg in foodstuffs and plants used for therapy have to be controlled. Thus, PTWI (Provisional Tolerable Weekly Intake) values for adults have been set up as: Cd, 7; Hg, 5; and Pb, 25 in $\mu\text{g}/\text{kg}$ body weights. The Recommended Dietary Allowance (RDA) for other elements also has been estimated for safety and adequate daily intakes for adults as follows: Cr, 0.6-3; Mn, 26-60; Se 0.9; and Zn 190 in $\mu\text{g}/\text{kg}$ body weights. Using these standards therefore, the elemental compositions (in mg/g) of *H. madagascariensis* stem bark pellet sample show the presence of various trace elements of different concentrations that could produce toxicity if administered in large quantity. It also shows that the plant contain important elements that are needed for growth and development, aid the prevention and healing of diseases in man and animals. For example Fe and Ca, are available in very moderate to very high concentrations respectively. This is very

significant, as Fe and Ca are known to enhance the qualities of blood, bones, teeth and blood formation and also of cardiac function. They also play a predominant role in oxygen and electron transport. The large amounts of Fe and Ca in *H. madagascariensis* stem bark shows that it could be specifically useful in the treatment of diseases and disorders like: malaria, anemia in pregnant mothers at a very low dose, as its main medicinal activities are as an anti-infective (against malaria, trichomonads, bacterial, fungi) (Okoli et al., 2002, Iwalewa et al., 2008a), anti-anemia, (Erah et al., 2003), bleeding and amenorrhea (Duke and Ayensu., 2002). For example, the red juice from the plant leaves and stem bark are reputed for arresting post-partum or post-abortual bleedings in Sierra Leone, while the unopened buds are equally reputed for treating puerperal infection in Liberia (Olagunju et al., 2000). Jubi formula, a herbal preparation made from three medicinal herbs (*Parquetina nigrescens*, *Sorghum bicolor* and *H. madagascariensis*) has been successfully used in the treatment of anemia in humans and in the treatment of *Trypanosoma brucei brucei*-induced anemia in rabbits (Erah et al., 2003). The presence of these elements could also serve as ingredients that favor its antioxidant effect, antimalarial, antitrichomonal and anti-amoebic activities of *H. madagascariensis* stem bark (Tona et al., 2000; Iwalewa et al., 2008a,b). The beneficial effects of copper to plants and animals are numerous. Copper as a trace element has an inhibitory effect on edema and inflammation. The effect of copper on resistance to bacterial and viral infection in ruminants has been highlighted by Suttle and Jones (1989). Likewise, several investigators have explained the effect of iron overload on microbial infections. Van

Asbeck et al. (1982) demonstrated that iron has a deleterious effect on the phagocytic capacity of both monocytes and granulocytes *in vitro*. Mencacci et al. (1997) indicated that iron overload may negatively affect CD4 Th1 development in mice with candidiasis, a function efficiently restored by therapy with deferoxamine, an iron chelator. This report of *H. madagascariensis* stem bark is a milestone as it shows that the elemental constituents of this plant is a source of dietary and food supplement for both animals and man especially when taking in very small doses. It could also be a pointer to how the plant produces its biological activities.

Several workers like Adams, 1953; Beutler and Blaisdell, (1958) have used biochemical indices like Hb concentration, RBC count and PCV to evaluate the state of iron deficiency anemia, hence, the use of these indices in this study. According to Dhawan and Srimal (1997), the normal values for PCV, RBC count and Hb in normal control rats are 40–52%, $6.5\text{--}9.0 \times 10^4$ and 60.4–76.8 g/dL respectively. From the results of malaria induced anemia groups, it is glaringly clear that the constituents of *H. madagascariensis* stem bark have elevated and contributed to the depleted constituents of the blood. The result of our study also indicated that anemia induced by malaria parasites in this experiment is not due to vitamin B₁₂ and artemether as anti-malarial agent has little effect on anemia induced by rodent malaria parasites. Artemether and vitamin B₁₂ could not effectively reverse the anemic condition by increasing the number of RBC. The inability of artemether and vitamin B₁₂ to reduce anemia could be possibly attributed to the various pathophysiological effects produced during malaria infection in mice. It has been established that the degree of anemia always correlates well with increase in parasitemia and levels of hemotocrit (Iwalewa, 1995), therefore as indicated in this study, the effect of extract on anemia induced by malaria shows that anemia was reversible. There was a continuous reduction in the value of PCV, RBCs and hemoglobin contents in the control group between D0 and D6, however in all the treated groups between D3 and D6 of infection, there was increase in the value of PCV, RBCs and hemoglobin. This is an indication that the elemental constituents in the extract has the ability to effectively inhibit the growth of the parasite in the RBC as previously reported in our earlier study (Iwalewa et al., 2008a) and couple with the fact that the extract contains Fe, the PCV, RBCs and hemoglobin contents could have increased.

Oxidative stress on erythrocytes is considered an important mechanism of hemolysis. Disruption of membrane integrity arises from fragility, dehydration as well as increased production of reactive oxygen species. Chronic hemolysis leads to loss of hemoglobin. These metabolic changes lead to depletion of essential

nutrients and micronutrients which are required for proper cell function. Since non-parasitized erythrocytes are also destroyed, and in acute malaria, the presence of pro-oxidants is well established, the released constituents at the extracellular environment of the erythrocyte may be a contributor to the oxidative stress.

Micronutrient deficiencies and infectious disease often coexist and show complex interactions leading to mutually reinforced detrimental clinical effects especially in underprivileged people of developing countries, particularly in rural regions. Several micronutrients such as trace elements (Zn, Fe, Se) modulate immune function and influence the susceptibility of the host to infection. Some trace elements have been found beneficial in the control of anemia under this condition. These include Fe, Cu, Zn and folate (Prasad, 1999). There have been a linkage or a direct correlation between antioxidants effect of trace elements, malaria and anemia. Deficiencies of trace elements do have negative impacts on malaria and anemia (Okochi and Okpuzor, 2005). Therefore, in this study, the presence of these trace elements in *Harungana* could serve as antioxidants, hence contributed to the plant anti-anemic and anti-malarial properties. This however, corroborates our earlier findings (Iwalewa et al., 2008a,b). In conclusion, this study shows that *H. madagascariensis* stem bark extract constituents' exhibit anti-anemic activity. The effects in both malaria and phenylhydrazine-induced anemia are suggested to be influenced with the presence of the macro (K, Ca, Mg, Cr) and micro (trace) elements (Cu, Fe, Mn, Mo, Ni, Zn) detected in different concentrations that could produce toxicity if administered in large quantity.

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