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Adverse Haematological Changes in Plasmodium Infected Mice Following Treatment with *Azadirachta indica* (Neem) Leaf Extract

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Authors' contributions

This work was carried out in collaboration among both authors. Author HDA designed the study, wrote the protocol and did part of the literature search. Author ISE managed the animals, collected all data, performed the statistical analysis and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Background: *Azadirachta indica* (neem) is an effective natural remedy for malaria treatment in some tropical regions of Africa. This study investigated the impact of *Azadirachta indica* leaf-extract on some haematological indices of *Plasmodium berghei-berghei* infected mice.
Materals and Methods: Twenty-five (25) mice weighing between 19 g and 31 g were randomly selected into five (5) groups of 5 animals per group. Groups 1, 2 and 3 were inoculated on day 0, intraperitonealy about 1% ($4.5x10^4$) *Plasmodium berghei berghei* parasitized red blood cells. Groups 1 and 2 were treated with 50 mg/kg and 75 mg/kg of the extract respectively by oral gavage, while group 3 animals were given normal saline. Groups 4 and 5 were not infected but group 4 was treated with 75 mg/kg of the extract and 5 served as normal control mice. Treatment lasted for 14 days. Whole blood was obtained for haematological analysis using Sysmsec® Automated Haematology Analyzer KX-21N.

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Results: The result showed significant reduction in red blood cell count (RBC: p = 0.000), packed cell volume (PCV: p = 0.000) and haemoglobin concentration (HB: p = 0.000) of *P. berghei berghei* infected mice following *A. indica* leaf-extract administration. The mean cell haemoglobin (MCH: p = 0.006) and mean cell volume (MCV: p = 0.024) were significantly higher when compared to uninfected and untreated control mice. The red cell indices (RBC count, PCV and HB) animals that were infected without extract treatment were significantly higher than those of infected mice that received the extract. Extract treatment to uninfected mice, however, did not show any significant change in these parameters when compared with untreated control animals. Total white blood cell count (WBC: p = 0.005), neutrophils (p = 0.024) and lymphocytes (p = 0.001) were significantly higher in infected treated mice than those without extract treatment. Administration of extract to uninfected mice did not cause any significant change in total white blood cell counts and the differential counts. However, eosinophil (p = 0.214) and platelet (p = 0.152) counts of infected mice receiving extract treatment were not significantly different from those of uninfected control. **Conclusion:** We therefore conclude that treatment of malaria with extract of *A. indica*, though may be effective as anti-malaria and could prevent malaria associated platelet changes, may contribute to malaria associated anaemia. Care should, therefore, be taken to follow up the extract treatment with blood building agents.

Keywords: Azadirachta indica; red cell indices; white blood cells; platelet counts; plasmodium malaria mice.

1. INTRODUCTION

Changes in haematological indices resulting in diverse degrees of anaemia are common findings in many disease conditions including malaria, hookworm infestation, chronic inflammatory and immune disorders, cancer and chronic renal disease. The mechanisms of induction of anaemia vary with the disease conditions. The pathophysiologic mechanism for malaria-induced anaemia and other complications involve destruction of infected erythrocyte, liberation of parasite and erythrocyte materials into circulation and the host reactions to these events [1]. Once in the blood stream, *plasmodium* invades liver and red blood cells and makes more copies of itself. Eventually, as red cells break and free plasmodium to infect other red cells and as the body's immune system works to kill infected cells, the total number of red blood cells drops causing anaemia [2].

Also, plasmodium infected erythrocytes sequester in microcirculation of vital organs interfering with micro-circulatory flow and host tissue metabolism. The infected red blood cells rosette and stick to endothelial cells lining the blood vessels [3]. Several proteins including plasmodium falciparum erythrocyte membrane protein I (PFEMP1) form knobs on the surface of the infected red cells and bind to ligands including CD36 thrombospondin, VCAm-1, ICAM-1 and E-selections displayed on the surface of endothelial cells [4]. Ischaemia results from impaired circulatory blood flow in the affected vessels supplying vital organs, thus leading to most of the manifestations seen in severe malaria. Merozoite surface antigens released from infected red cells induce the production of TNF-α, interferon (IFN) and IL-1, by the host cells and these cytokines suppress the production of red cells, increase fever and induce nitric oxide syntheses leading to tissue damaged and increased expression of endothelial receptors for PfEMP-1 [5].

Azadirachta indica (Neem) a large green tree of the family maliaceal, is believed to originate from Assan and Burma in South Asia [6]. It is one of the most useful medicinal plants used traditionally as remedies for various diseases [7]. It has been speculated by traditional medicine practitioners to be effective for treatment of activities, leprosy, malaria, typhoid fever, respiratory disorders, constipation, cancer and chronic syphilis sores. Scientific studies have also demonstrated some specific pharmacological properties of neem leaf-extract; for instance, hypolipadaemic, antioxidant and anti-diabetes activities of the leaves extract have been reported in diabetes rat models [8-10]. Immunostimulatory [11,12]; anti-inflammatory [13,14] and hepatoprotective [15] activities have also been shown in various experimental models. Traditional use of neem leaf-extract for the treatment of malaria had long been observed and some experimental evidences of its antimalarial activities have been documented [16,17].

Although vector control and use of chemoprophylates agents such as chloroquine, quinine, amodiaquine. Sulfadoxine, pyrimethamine and artemicinine, either individually or in combination, are common preventive/curative measures against malaria infection; attention is also being focused on the use of effective and safe natural remedies as alternative strategies. This has been encouraged with a view to bringing malaria treatment closer to the poor rural dwellers of the African societies who can hardly afford or access conventional anti-malaria drugs; and to cope with the emergence of high rate of drug resistant strain of malaria parasite and cases of adverse drug reactions. *Azadirachta indica* (neem) is one of such popular natural remedies for malaria treatment in tropical regions of Africa, and it has been shown to be effective in this regard. However, the effects of the plant extracts on some blood parameters have not been closely examined. This study therefore is aimed at investigating the impact of *Azadirachta indica* leaf-extract on some haematological indices of *plasmodium berghei-berghei* infected mice model.

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Materials

The fresh leaves of neem (*Azadirachta indica*) were harvested from the Endocrine Research farm, University of Calabar, Calabar. The samples were taken to the Department of Pharmacognosy, University of Uyo, Uyo, where it was authenticated. The fresh leaves of neem were collected and selected to remove the dead ones. Leaves were washed with distilled water and sun-dried to remove the water. The leaves were blended using an electric blender and soaked in ethanol for 24 hours. The mixture was filtered and the residues were discarded. The filtrate was concentrated in a water bath at 50℃.

2.2 Determination of Extract Concentration by Gravimetric Method

The stock solution was stirred to mix. 2mls aliquot was drawn from the stock solution and oven dried in a beaker of known weight at 50℃. The beaker now containing the residue was then reweighed. The difference in weight of the empty beaker and when it contained the residue indicated the weight of the residue.

2.3 Experimental Animals

Twenty- five (25) mice were obtained from Pharmacology and Toxicology departmental animal house, University of Uyo. They were caged in a well ventilated room with 12 hours light-dark cycle. They were fed on commercial diet (Guinea Feed Ltd, Benin) and were also given tap water for 7 days during which they acclimatized.

2.4 Induction of Malaria and Parasite Inoculation

Fifteen (15) mice weighing between 19 g and 31 g were randomly selected for induction of malaria, they were assigned to three groups with 5 animals per group. Chloroquine sensitive *Plasmodium berghei-berghei* (animal parasite that causes the malaria) obtained from the National Medical Research Institutes, Abuja were maintained in mice. The parasite donor mice contained 5% ($22.5x10^4$) *Plasmodium berghei-berghei* parasitized erythrocytes. The parasite donor blood was diluted with normal saline in proportion such that each mouse was inoculated on day 0, intraperitonealy about 1% ($4.5x10^4$) *Plasmodium berghei berghei* parasitized red blood cells [18]. The animals were confirmed to be infected with malaria 72 hours after the inoculation.

2.5 Experimental Groups

Group 1: 5 infected animals receiving 50 mg/kg of the extract orally.

Group 2: 5 infected animals receiving 75 mg/kg of the extract orally.

Group 3: 5 malaria infected animals that did not receive any extract.

Group 4: 5 normal animals receiving 75 mg/kg of the extract orally.

Group 5: 5 normal animals receiving no extract.

2.6 Collection of Samples for Haematological Analysis

At the end of the 14 days, they were then euthanized under chloroform vapour and sacrificed. Whole blood was collected via cardiac puncture using sterile syringes and needles. The whole blood was emptied into EDTA containers preparatory to haematological (full blood count) analysis. Full blood counts including PCV, Hb, RBC, WBC, platelet count, differential WBC (lymphocytes and mixed), and red cell indices

(MCHC, MCH and MCV), were estimated using the Sysmsex® Automated Haematology Analyzer KX-21N, Sysmex Corporation, Kobe-Japan. The pre-diluted (PD) sample method was used where blood was diluted manually, and then fed into the transducers. The transducer chamber has a minute hole called the aperture. On both sides of the aperture, there are electrodes between which flows direct current. Blood cells suspended in the diluted sample pass through the aperture, causing direct current resistance to change between the electrodes. As direct current changes, the blood cell size is detected as electric pulses. Blood cell count is then calculated by counting the pulses, and a histogram of blood cell sizes is plotted by determining the pulse sizes. Also, analyzing a histogram makes it possible to obtain various analysis data including differential whole blood count, red cell indices and derived values.

The procedures used in this study were approved by the Postgraduate School Research Ethical Committee, University of Uyo.

2.7 Statistical Analysis

Data are presented as Mean ± standard deviation. Differences between means were compared employing student's t-test and ANOVA post hoc, a probability of $p < 0.05$ was considered significant.

3. RESULTS

Haematological changes associated with administration of *A. indica* leaf-extract to *Plasmodium berghei berghei* infected mice were investigated. The effects of neem leaf - extract treatment of infected and uninfected mice on red blood cell indices are shown in Table 1. The result showed that administration of *A. indica* leaf-extract to *P. berghei berghei* infected mice caused a significant reduction in some red blood cell indices namely: Red blood cell count (RBC: $p = 0.000$), packed cell volume (PCV: $p = 0.000$) and haemoglobin concentration (HB: $p = 0.000$) while mean cell haemoglobin (MCH: $p = 0.006$) and mean cell volume (MCV: $p = 0.024$) were significantly higher when compared to uninfected and untreated control mice. Animals that were infected without receiving treatment with the extract also showed significant decrease in RBC count ($p = 0.004$), PCV ($p = 0.006$) and HB concentration ($p = 0.005$) but not in MCH ($p = 0.329$) and MCV ($p = 0.357$) when compared with control. However, the red cell indices (RBC count, PCV and HB) in this group were significantly higher than those of infected mice that received the extract. Extract treatment to uninfected mice did not show any significant change in these parameters when compared with untreated control animals.

Changes in white blood cell indices following *A. indica* leaf-extract treatment of infected and uninfected mice were presented in Table 2. Total white blood cell count (WBC: $p = 0.005$), neutrophils ($p = 0.024$) and lymphocytes ($p = 0.001$) were significantly higher in infected mice that received extract treatment than those without treatment. Administration of extract to uninfected mice did not cause any significant change in total white blood cell counts and the differential counts. However, eosinophil ($p = 0.214$) and platelet ($p = 0.152$) counts of infected mice receiving extract treatment were not significantly different from those of uninfected control but eosinophil ($p = 0.000$) was rather higher and platelet ($p = 0.001$) was lower in infected mice that received no treatment. Significant positive correlation existed between parasite-induced eosinophilia and total white blood cell count ($r = 0.993$, $p = 0.001$), neutrophil ($r = 0.898$, $p = 0.050$) and lymphocytes ($r = 0.994$, $p = 0.004$) in plasmodium infected mice that were not treated with extract. Significant negative correlation was also observed between eosinophil and platelet counts ($r = 0.818$, $p = 0.007$) in this group of mice.

4. DISCUSSION

Unusual haemolytic reactions are known to occur in individuals following administration of different antimalarial drugs. Agents such as quinine, chloroquine, primaquine fansidar and coartem are quite notable among others [19]. Other studies have also demonstrated dose dependent increase in blood methaemglobin concentration and increasing osmotic fragility of erythrocytes following administration of fansidar, halofanthrine, quinine, chloroquine and coartem [20,21]. All these drugs were shown to generate rapid accumulation of reactive oxygen species (ROS) that overwhelmed the antioxidant defense capacities of the erythrocyte and causes depletion of erythrocyte glutathione concentration and red cell lysis. Also, plasmodium infected erythrocytes are subsequently destroyed in the lymphoid tissues by cytotoxic T-lymphocytes and natural killer cells as blood is passing through these organs thereby contributing to malaria associated anaemia [2].

Table 1. Changes in red cell indices of plasmodium infected and uninfected mice following treatment with *A. indica* leaf-extract

Group	Pcv (%)	Hb g/l	RBC (X10$^{12/L}$)	MCHC (g/L)	MCH (pg)	MCV (fL)
1:Malaria±50 mg/kg *A. indica*)	15.40±2.23*	51.40±1.12*	1.34±0.11*	343.22±0.38	38.82±7.79*	113.14±23.37*
2:Malaria infection±75 mg/kg	15.0 00±2.00*	50.40±7.13*	1.34±0.12*	335.89±9.58	37.70±4.53*	112.16±13.69*
3:Malaria infection only	24.00±0.71*[a]	79.20±1.30*[a]	2.66±0.15*[a]	330.28±5.96	29.86±1.60	90.54±6.34
4:Normal mice±75 mg/kg *A. indica*	35.80±3.84	119.40±5.57	4.06±0.24	333.72±66.92	29.54±1.35	88.40±3.90
5:Normal mice (Control)	37.60±1.14	125.40±3.91	4.42±0.14	334.18±1.23	28.46±0.86	86.48.±2.31

*Data are expressed as mean ± SD. n = 5 mice per group. * significant difference at p < 0.05 compared with control. [a] significant difference at p < 0.05 compared with extract treated infected mice*

Table 2. Changes in white blood cells and platelets of plasmodium infected and uninfected mice following treatment with *A. indica* leaf-extract

Group	WBC x 10^3/µL	Neut x 10^3/µL	Lymp x 10^3/µL	Eosin x 10^3/µL	Platetes x 10^3/µL
1: Malaria±50 mg/kg *A. indica*)	22.54±0.95*	4.56±0.24*	18.88±1.22*	0.24±0.020	116.20±8.14
2: Malaria infection±75 mg/kg	28.04±3.77*	6.16±0.84*	21.88±2.93*	0.25±0.038	130.40±6.58
3: Malaria infection only	14.80±1.35*[a]	2.32±0.25*[a]	12.42±1.03*[a]	0.59±0.056*[a]	95.20±6.72*[a]
4: Normal mice±75 mg/kg *A. indica*	8.26±1.29	1.88±0.47	7.10±1.07	0.21±0.021	128.80±6.22
5: Normal mice (control)	6.42±1.78	1.24±0.55	5.50±2.42	0.20±0.03	128.40±6.19

*Data are expressed as mean ± SD. n = 5 mice per group. * significant difference at p < 0.05 compared with control. [a] significant difference at p < 0.05 compared with extract treated infected mice*

In this present study, we found that treatment of *P. berghei berghei* infected mice with leaf extract of *A. indica* caused significant reduction in red blood cell indices namely; total red cell count, PCV, HB concentration, and increase in MCH and MCV compared to uninfected mice that received no extract. Also infected mice that received no treatment showed a less severe anaemia than those treated with the extract while uninfected mice which were treated with the extract did not show significant change in the red cell indices compared to normal mice that received no treatment. These findings suggest that treatment of infected mice with *A. indica* leaves may enhance selective destruction of infected red blood cells in the affected mice groups while the uninfected red cells were spared.

A. indica is a well-known medicinal plant with scientific evidence of antimalarial activities [17]. The mechanism of its antiplasmodial activities is not fully understood, but had been shown to demonstrate significant immune-stimultory and anti-inflamatory antivities [11,12]. Acetone-water neem leaf extract having antimalarial activity had been shown to inhibit the adhesion of malaria parasite-infected erythrocytes to endothelial cells [17]. Therefore, prevention of cytoadherence of infected erythrocyte to vascular endothelium increase the infected red cell contact with the cytotoxic T-lymphocyte and natural killer cells in the lymphoid tissue, thus resulting in increased destruction of parasitised cells and reduced parasite asexual reproduction in red cells. The extract is known to have significant anti-inflammatory activity as demonstrated by its ability in suppression of neutrophils and macrophages activation [14]. The anti-inflammatory effect can also be related to decreased expression of adhesion molecules by neutrophils and vascular endothelium and hence decreased cytoadherence.

The pathologic process of malaria infection and its complications involves cytoadherenece and sequestration in micro circulation of vital organs of the body. Erythrocytes containing mature form of plasmodium express several surface proteins (including PfEM-PI) which form kobs with ability to adhere to microvascular endothelium and thus disappear from the circulation, remaining stuck until they rupture at merogony (schizogony) to exacerbate the infection by infecting new red blood cells. The consequences of microcirculatory obstruction are activation of the vascular endothelial cells and reduced oxygen and substrate supply, leading to anaerobic glycolysis, acidosis and cellular dysfunction [2,3].

The mechanism by which *A. indica* leave extract causes severe anaemia in plasmodium infected mice, may therefore be due to decreased cytoadhesion of parasitized red cells to endothelium. This is particularly likely because administration of the extract to non-infected mice did not cause anaemia and the levels of anaemia in untreated plasmodium infected mice were not as severe as those treated with the extract. As indicated by the present study, the anaemia caused by malaria parasites alone without extract treatment is normochromic and normocytic in nature which is characteristic of mild haemolysis. However, treatment of the infected mice with *A. indica* leaf extract presented a macrocytic and slight hyperchronic picture as evident by increase MCV and MCH. This may be due to increased release into circulation of reticulocytes as the body is responding to the severe anaemic condition by increasing production of new red bloods cells.

We also observed that the white blood cells increased significantly in infected mice that were treated with the extract than in those that were not treated and those that were not infected by plasmodium but treated with the extract. This suggest an enhance activation and proliferation of the immune cells by the extract following antigenic challenge such as *Plasmodium* infection. Baral and Chattopadhyay [12] had shown that neem leaf extract mediated higher immune activation. Activated lymphocyte (including T- lymphocyte and Natural killer cell) in the lymphoid organ and in circulation could contribute to the excessive and selective destruction of red cells infected by the parasite. Most conventional anti-malarial drugs cause red cell destruction by increased generation of ROS and depletion of anti-oxidant defense molecules [19], this mechanism might not generally be true for *A. indica* extract because earlier report had shown that the extract has significant anti-oxidant activities probably owing to its high concentration of polyphenols, such as flavonoids [10], but may be related to its ability to inhibit cytoadherence of infected cells to endothelium.

The eosinophil counts of infected mice receiving extract treatment were not significantly different from those of control mice but the untreated infected mice should significantly higher eosinophil count relative to control. In the untreated mice, the increase in eosinophil may

be a normal body's response against parasitic infection. However, the administration of neem leaf-extract ameliorates this effect by preventing activation and proliferation of the eosinophil through the same mechanism as indicated by Kaur and his colleagues [14]. This may further be supported by the significant positive correlation observed in this study between parasite-induced eosinophilia and total white blood cell counts, neutrophil and lymphocytes in plasmodium infected mice that were not treated with the extract.

Platelet activation and increased tendency for intravascular coagulation is characteristic of plasmodium malaria. Our studies showed increased platelet consumption in untreated infected mice as evident by significant decrease is platelet number compared to control mice. However, infected mice that were treated with the extract did not show significant reduction in platelet number. This finding suggests that the extract can prevent platelet activation and aggregation which can go a long way to reducing malaria associated coagulation complications. The anti-inflammatory property of the leaf extract may be responsible for this platelet affects.

5. CONCLUSION

We therefore conclude that treatment of malaria with extract of *A. indica*, though quite effective as anti-malaria and could prevent malaria associated bleeding disorders, may contribute to malaria associated anaemia and care should be taken to follow up the extract treatment with blood building agents.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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