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Inhibition of *Plasmodium berghei* growth by alkaloid extract of *Phyllanthus amarus* in mice increased haem level and stabilized erythrocyte membrane

Opajobi A O

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Ojugbeli E T

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Uzuegbu U E

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Elu C O

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Toloyai P Y

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Awhin P E

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Oshiegbo W

Department of Biochemistry, Faculty of Science, Delta State University, Abraka, Nigeria

Onyesom I

Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria

Corresponding author email: ionyesom@delsu.edu.ng

Abstract--Objectives: The *Plasmodium* parasite is known for its ability to destabilize the red cell membrane causing the release of free haem which is converted to haemozoin as haem is toxic to parasite. In combination with other factors, accumulation of haemozoin is associated with anaemia which manifest in severe malaria. Compounds directed at preventing erythrocyte attack and increasing haem concentrations could serve as new sources of antimalarials. Therefore, this study assessed the ability of *Phyllanthus* (*P.*) *amarus* alkaloid extract, a phytochemical, already reported to possess antimalarial activity, in reducing erythrocyte deformity and preventing parasite multiplication by increasing haem concentrations. Methods: The study involved measurement of parasite clearance, changes in haematological indices and degree of erythrocyte membrane stabilization (using heat-induced and hypotonic-solution induced destabilization assays) in *Plasmodium* (*P.*) *berghei* infected mice treated with alkaloid extract of *P. amarus* using documented methods. Results: Infection of experimental mice with *Plasmodium berghei*, induced malaria with evidence of anaemia (reduced PCV and Hb), reduction in haematological indices (RBC, WBC, platelets, lymphocytes, and eosinophil), increased serum haemozoin and haem concentration and destabilization of erythrocyte membrane. However, treatment with alkaloid extract of *P. amarus* reversed the order in a dose-dependent pattern and in a manner that compared well with the standard drug treatment, Lonart®DS. Conclusions: Alkaloid extract of *P. amarus* demonstrated potent antimalarial activity with the ability to stabilize damaged erythrocyte membrane and inhibit parasites' haem conversion to haemozoin. Therefore, alkaloids of *P. amarus* could be pivotal in the discovery of novel and more efficient antimalarial agent(s).

Keywords--*Plasmodium berghei*, alkaloid extract, erythrocyte membrane.

Introduction

The pathogenesis of *Plasmodium* infection is characterized by erythrocyte assault caused by interactions between parasites and erythrocyte membrane, leading to destabilization of erythrocyte membrane which further progresses to a cascade of disruptive processes including loss of erythrocyte function, lysis of erythrocytes, increased haemozoin and haem concentrations, hematological abnormalities, anaemia, oxidative stress, cerebral malaria and sometimes death^(1,2). Antimalarials, therefore, targeted at preventing parasitic invasion by stabilizing erythrocyte membrane could curb the negative progression of malaria. Commonly used antimalarials, artemisinin combination therapy (ACTs), approved by the World Health Organisation as the front line for malaria chemotherapy, act by inhibiting the conversion of haem to haemozoin and destabilising the erythrocyte membrane^(3,4). Other antimalarials, quinine, artesunate and chloroquine, which have been reported as not effective due to parasite resistance, also act via this method^(5,6). Therefore, one could expect that parasites could also develop

resistance to ACTs. Tilley *et al*⁽⁷⁾, who already reports parasites resistance to artemisinin derivatives, further stated that after treatments, asexual parasites can become dormant in infected erythrocytes and may regrow after concentrations of antimalarials in blood, decline. Artemisinin resistance in *P. falciparum* reflects reduced ring stage susceptibility and manifests as slow parasite clearance. Therefore, the search for new drugs for malaria eradication is still needed.

The antiplasmodial activity of *Phyllanthus amarus*, the plant selected for our study, has been reported and confirmed by several authors⁽⁸⁻¹⁰⁾ and its activity has been reported to reside greatly in the alkaloid phytochemical of the plant^(9,10). However, its mechanism of action is not fully known. Nonetheless, its ability in stabilizing the erythrocyte membrane has only been reported by Chopade *et al*⁽¹²⁾. Olawale *et al*⁽¹³⁾, reports that aqueous extracts of *P. niruri*, a species of *Phyllanthus*, protects against severe malaria by protecting erythrocyte membrane from parasite invasion. Sufficient literature on the erythrocyte stabilization mechanism of *P. amarus* is scarce. Therefore, in our present study, we evaluated the potential of alkaloid extract of *P. amarus* in stalling the conversation of harm to haemozoin and stabilizing the erythrocyte membrane in *Plasmodium berghei* infected mice.

Materials and Methods

Collection of plant materials and preparation of alkaloid extracts

Leaves of *Phyllanthus amarus* were collected, washed and air dried at laboratory room temperature (28°C-32°C) and, thereafter, crushed using a laboratory blender (Kenwood, Japan). Alkaloid extract was prepared by immersing dried concentrate in 100 mL of ammonia for 1h. Thereafter, filtrate from mixture was again immersed in 500 mL of dichloromethane for 24h. Solid alkaloids, were obtained by concentrating residue from mixture⁽¹⁰⁾.

Experimental Animals, Grouping, and Infection

Donor mice already inoculated with parasites (*Plasmodium berghei* NK 65) were obtained from the Department of Parasitology, Nigerian Institute of Medical Research, NIMR, Yaba, Lagos State, Nigeria. Adult Swiss mice (about 8 week old, BALB/c albino strain; 22-28 g bwt) were purchased from Laboratory Animal Centre, LAC, Faculty of Basic Medical Sciences, FBMS, Delta State University, DELSU, Abraka, Nigeria. Thirty (30) mice segregated into six (6) groups (5 mice per group) were used for this study. The six groups included: three control groups; positive control (neither infected, nor treated), negative control (infected, not treated) and standard control group (treated with 20 mg/kg artemether and lumefantrine, Lonart®DS) and three test groups, infected and treated with varying doses (100, 200 and 300 mg/kg) of alkaloid extracts of *Phyllanthus amarus*. Experimental mice were infected with inoculum prepared by diluting 0.1 mL of infected blood obtained from infected (donor) mice, in 0.9 mL of PBS, pH 7.2. Dosage was selected based on previous studies⁽¹⁴⁾.

Parasite Clearance

After 72h of inoculation and infection confirmation, *P. amarus* alkaloid extract and standard drug were administered once daily to specific groups using intragastric cannula for four (4) days. At day 0, 3, 6, 9 and 12, blood smears were made to determine parasite clearance, by collecting blood from the cut tail tip of infected mice and stained with Giemsa. Slides were later viewed under the microscope (TH-9845, Serico, China) at x40 magnification. Infected and uninfected erythrocytes were counted and parasite clearance was calculated with formula by Olanlokun *et al*⁽¹⁵⁾:

$$\begin{aligned} \text{Parasite Clearance (\%)} &= (\text{Infected erythrocytes} \times \text{neg control}) \\ &\quad - [\text{infected erythrocytes} \frac{\text{test}}{\text{infected erythrocytes}} (\text{neg control})] \times 100 \end{aligned}$$

Hematological Assay

Haematological indices were assessed using documented methods⁽¹⁶⁾. After the sixth day of post treatment (ie the 12th Day), the mice were fasted overnight and then, weighed and euthanized. Thereafter, 5ml of blood was collected from each animal by cardiac puncture with a sterile syringe and needle into EDTA treated screw-cap sample bottles. Full blood counts including, packed cell volume (PCV), haemoglobin (Hb), red blood cells (RBC), platelets, white blood cells (WBC), lymphocytes, neutrophils, monocytes and eosinophil were estimated using an hematological analyzer (Sysmex® Automated Haematology Analyzer KX-2IN, Sysmex Corporation, Kobe, Japan).

Haem Content

About 10 µL of blood was added to a mixture of 250 µL of 10% sodium dodecyl sulfate and 250 µL of 1 M sodium hydroxide and left at room temperature for 2 h. Haem content was measured at 404 nm⁽¹⁷⁾.

Haemozoin Content

About 10 µL of blood, lysed in 0.008 % saponin was spun at 18 000 rpm in a centrifuge. Resulting supernatant was removed and pellet washed thrice. Thereafter, pellet was incubated at 37°C for 12 h. Tubes were centrifuged again at same speed and pellet was hydrolyzed to monomers with 1M NaOH. Absorbance was read at 404 nm. Haemozoin content was expressed as micromole haem/mL of blood⁽¹⁸⁾.

Erythrocyte Membrane Stabilization Assay

Blood samples, collected into EDTA bottles using methods stated above, were centrifuged and blood cells were washed thrice with a solution (154 nM NaCl, 10 mM sodium phosphate buffer) and centrifuged for 10 min at 5200 rpm to obtain erythrocyte suspension⁽¹⁹⁾.

Heat-Induced Membrane Dis-Stabilization

Dis-Stabilization assay was carried out according to methods stated by Olanlokun *et al*⁽¹⁵⁾. Assay mixture consisted of 0.5 mL hypo saline, 1.0mL sodium phosphate buffer (0.15M, pH 7.4), the varying volume of iso-saline and 0.5mL mice erythrocyte suspension. Varying concentrations of extracts and standard drug were added to above mixture to obtain the test groups and standard control group, respectively. Negative control was without standard drug or extracts and positive control contained above mixture without erythrocyte suspension, standard drug or extract. Mixture was incubated at 56°C for 30 min. After cooling, mixture was centrifuged at 3000 rpm for 5 min. Absorbance of resulting supernatant was read at 560 nm. The percentage membrane stability activities were estimated with the formula:

$$100 - \frac{\text{Absorbance test} - \text{Absorbance negative control}}{\text{Absorbance negative control}} \times 100$$

Hypotonic Solution-Induced Membrane Dis-Stabilization

About 0.5 mL of erythrocyte suspension was mixed with a solution of 5ml NaCl (50 mM) and 10 nM sodium PBS (pH 7.4). *P. amarus* extract at different concentrations was added to the mixture. Mixture was left to stand for 10 min and thereafter, centrifuged for 10 min at 5200 rpm. Positive control consisted of erythrocyte suspension in hypotonic buffered saline. The resulting supernatant's absorbance was measured at 540 nm⁽¹⁹⁾.

Statistical Analysis

Data were expressed as the mean $\pm$ SEM. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Turkey's post hoc (one-way) using Graph Pad prism (6.0). The level of significance was set at $P < 0.05$.

Results

Table 1 shows the percentage of parasite clearance and haematological indices in *Plasmodium berghei* infected mice treated with varying doses (100, 200, 300 mg/kg) of *Phyllanthus amarus* infected mice and comparing the values obtained to mice in positive and negative control groups and standard groups. As shown by values in Table 1, comparing negative control group to test groups (groups treated with *P. amarus* alkaloid extract) and standard control group, parasitaemia in experimental mice had declined significantly ($p < 0.05$) in a dose dependent manner. Comparing parasitaemia reduction and percentage of parasite clearance in test groups to standard control, groups treated with 200 and 300 mg/kg of *P. amarus* alkaloid extract showed similar activity ($p > 0.05$) with standard control group.

From Table 1, comparing positive control group to negative control, there was a significant reduction ($p < 0.05$) in PCV, haemoglobin, red blood cells, white blood cells, platelets, lymphocytes, eosinophil's and a significant increase ($p < 0.05$) in neutrophil, haemozoin and haem concentrations. However, there wasn't

significant changes in monocyte count. Improvement in PCV following treatment, was greatest for mice treated with 300 mg/kg of *P. amarus* alkaloid extracts, even showing significantly ($p<0.05$) higher increase than mice treated with standard drug and compared well with positive control ($p<0.05$). However, mice treated with 200 mg/kg of *P. amarus* alkaloid extracts showed similar ($P>0.05$) impact with standard group and mice treated with 100 mg/kg *P. amarus* alkaloid extract presented no ameliorating effect on PCV, showing statistically similar ($P>0.05$) values as negative control.

Comparing improvement of some parameters; Hb, WBCs, lymphocytes, and neutrophils followed the same trend. Mice treated with 200 and 300 mg/kg of *P. amarus* alkaloid extract showed a significant dose-dependent improvement in these parameters when compared with negative control mice ($P<0.05$). These groups also showed similar activity with the standard drug and positive control groups ($P>0.05$). However, test group treated with lowest dose of extract (100 mg/kg) showed no significant improvement when compared with negative control group ($P>0.05$).

All test groups and standard control groups showed similar ($p>0.05$) effect in improving RBC concentrations following groups malarial infection and RBC indices obtained in these groups showed no significant difference ($p>0.05$) with indices in positive control group. Reduced platelet count following parasite infection was alleviated by administration of alkaloid extract of *P. amarus* as there was a significant dose dependent increase following treatment. From Table 1, there was a significant ($p<0.05$) dose dependent increase in platelet concentrations of *P. amarus* alkaloid extract treated group when compared with concentrations in all control groups. Eosinophil improvement following parasite reduction was achieved only by mice groups treated with 100 and 200 mg/kg of *P. amarus* alkaloid extract and compared well with positive control group ($p>0.05$). Standard group, group treated with highest doses of extract and negative control group recorded similar levels of the indices ($p>0.05$).

There was a significant reduction ($p<0.05$) in haemozoin concentration in all test groups and standard groups when compared with negative control group. However, only group treated with 100 mg/kg of extract showed statistically similar ($p>0.05$) activity with positive control. Groups treated with 200 and 300 mg/kg of extract showed similar activity with standard group. Haem concentrations, however, further increased with *P. amarus* alkaloid extract and standard drug treatments. No significant changes in concentration of monocytes were observed among all groups ($P>0.05$).

Table 1: Parasite clearance and associated changes in haematological indices of *Plasmodium berghei* infected mice treated with alkaloid extract of *Phyllanthus amarus*

Indices	Positive	Controls Lonart (20mg/kg)	Negative	Alkaloid extract (mg/kg)		
				100	200	300
Parasitaemia (%)	-	2.0±0.6 ^a	35.0±6.3 ^c	6.0±1.3 ^b	4.0±1.2 ^a	3.0±0.9 ^a
Parasite Clearance (%)	-	89.5±8.4 ^a	0.0±0.0 ^c	70.0±5.8 ^b	81.0±6.3 ^a	84.6±6.8 ^a
PCV (%)	41.6±1.9 ^a	33.7±2.6 ^b	25.2±1.8 ^c	28.2±1.6 ^c	36.3±2.2 ^b	38.6±2.3 ^a
Haemoglobin (g/dl)	12.5±0.8 ^a	10.2±2.5 ^a	7.9±0.7 ^b	8.3±0.6 ^b	10.8±1.4 ^a	11.4±1.8 ^a
Red blood cells (x10 ¹² /L)	7.3±0.5 ^a	6.7±1.3 ^a	5.1±0.8 ^b	7.8±1.1 ^a	6.9±1.3 ^a	7.1±0.9 ^a
Platelets (x10 ³ /L)	470.2±60.1 ^a	493.4±58.1 ^a	410.3±48.2 ^b	680.4±70.3 ^d	731.6±61.3 ^a	588.3±58.6 ^c
White blood cells (x10 ⁹ /L)	6.3±1.3 ^a	5.5±0.9 ^a	3.9±0.8 ^b	4.2±0.7 ^b	5.3±0.6 ^a	5.7±1.1 ^a
Lymphocytes (%)	86.3±21.2 ^a	81.7±9.1 ^a	51.4±10.2 ^b	65.6±11.3 ^b	77.4±12.4 ^a	80.3±11.2 ^a
Neutrophils (%)	30.6±2.4 ^a	32.5±3.6 ^a	38.2±3.1 ^b	41.2±4.4 ^b	35.6±4.1 ^a	32.6±3.7 ^a
Monocytes (%)	1.1±0.02 ^a	1.2±0.04 ^a	1.7±0.5 ^a	1.3±0.06 ^a	1.1±0.01 ^a	1.0±0.02 ^a
Eosinophil (%)	2.7±0.8 ^a	1.3±0.03 ^b	1.0±0.02 ^b	1.4±0.6 ^a	1.5±0.7 ^a	1.1±0.03 ^b
Haem (μmol/mL of blood)	8.3±0.6 ^b	9.6±0.7 ^a	9.0±0.6 ^a	9.5±0.5 ^a	11.2±0.3 ^c	13.4±0.8 ^c
Haemozoin (μmol/haem of blood)	0.12±0.03 ^a	0.07±0.01 ^b	0.28±0.03 ^c	0.13±0.03 ^a	0.08±0.01 ^b	0.05±0.01 ^b

Data are expressed as Mean ± SEM for n=5 mice/group. Values bearing different letter superscript in row are significantly different (p>0.05). Data were obtained after six days of post treatment period.

The effect of *P. amarus* on the maintenance of erythrocyte membrane integrity was assessed using heat-induced and hypotonic-solution models. Results from the assay showed that there was dose-dependent reduction in the percentage destabilization of the erythrocyte membrane of mice treated with *P. amarus* alkaloid extract when compared with control. However, in standard group, with increasing doses, there was a subsequent increase in percentage erythrocyte membrane destabilization.

Table 2: Destabilization of erythrocyte membrane of *Plasmodium berghei* infected mice treated with alkaloid extract of *Phyllanthus amarus*

Treatments (mg/mL)	Erythrocyte membrane destabilization (%)	
	Heat-induced	Hypotonic solution-induced
Alkaloid extract		
0.15	17±3	19±3
0.30	14±4	15±2
0.60	5±1	8±1
Standard drug (Lonart®DS)		
0.15	8±2	3±1
0.30	13±2	5±1
0.60	15±2	9±2
Blood control	150±6	54±6

Data are expressed as Mean ± SEM for n=5 mice/group.

Daily progression of parasitaemia in experimental mice is illustrated in Figure 1. The figure shows that at day 12, parasitaemia in experimental mice had declined

significantly in a dose-dependent manner with *P. amarus* alkaloid extract treated groups, showing significant differences when parasitaemia on day 12 were compared with those on day 3.

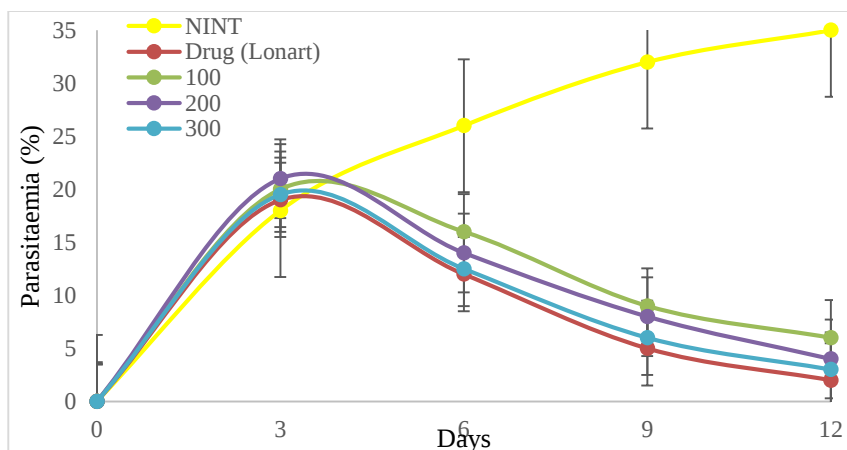


Figure 1: Daily progression of parasitaemia (%) in experimental mice induced by *P. amarus* alkaloid extract.

Day 0= Inoculation of mice with *P. berghei* malarial parasite. Day 3= Confirmation of parasitaemia and commencement of 4-day treatment with either the alkaloid extract of *P. amarus* at varying doses (100, 200 & 300 mg/kg) or standard drug (Lonart®DS) at a dose of 20 mg/kg. Day 6= End of the treatments. Day 9 & 12, third and sixth day post treatment, respectively.

Daily percentage of parasite clearance recorded on a 3 day basis is illustrated in Figure 2. There was a dose dependent increase in percentage of parasite clearance in *P. amarus* alkaloid extract treated groups, also presenting significant differences when percentage of parasite clearance on day 12 were compared with those on day 3.

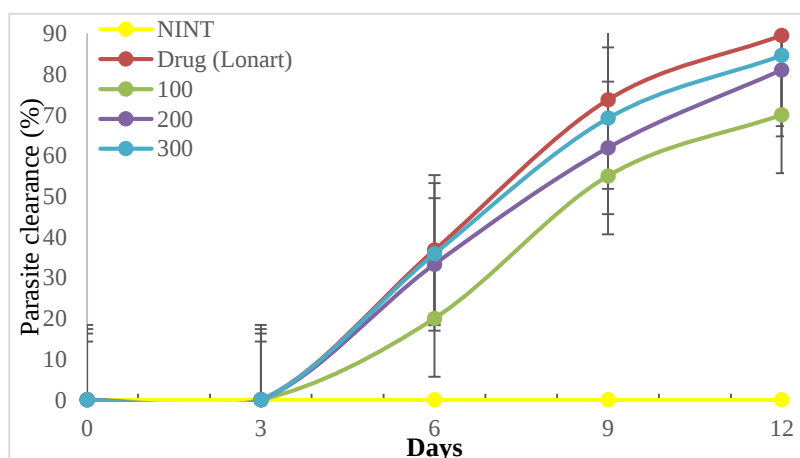


Figure 2: Dose-response curves for parasite clearance (%) induced by *P. amarus* alkaloid extracts in experimental mice.

Day 0= Inoculation of mice with *P. berghei* malarial parasite. Day 3= Confirmation of parasitaemia and commencement of 4-day treatment with either the alkaloid extract of *P. amarus* at varying doses (100, 200 & 300 mg/kg) or standard drug (Lonart®DS) at a dose of 20 mg/kg. Day 6= End of the treatments. Day 9 & 12, third and sixth day post treatment, respectively. The parasite clearance indicated is with respect to the corresponding Day 3 data that confirmed infection and success of inoculation.

Discussion

The most lethal and the most common form of malaria is caused by the *Plasmodium falciparum*⁽²⁰⁾. Its lethality is associated with the manifestation of severe forms of the infection, cerebral malaria and anaemia. The life cycle of the *Plasmodium* parasite in host, consists of two phases; the liver stage (sexual stage) and the blood stage (asexual stage)⁽²¹⁾. The blood stage which is the most target for antimalarials, begins with parasite invasion of erythrocytes⁽¹⁾. This is facilitated by parasite ligands, erythrocyte binding antigen (EBA) family of proteins⁽²¹⁾. Erythrocyte binding proteins; EBA175, EBL-1 and EBA-140 on *Plasmodium falciparum* merozoites and their receptors, glycophorin A, glycophorin B and glycophorin C, respectively on erythrocytes have been identified⁽²²⁾. Other major parasite ligands, reticulocyte binding proteins (Rh family) and the red cell anion transporter protein, have also been implicated in the initial interaction between the merozoite and the red cell^(22,23). *P. falciparum* merozoite binding to erythrocyte, activates a signaling pathway via a phosphorylation cascade, altering the properties of the erythrocyte membrane, thereby deforming it for invasion⁽²⁰⁾. Within erythrocytes, the parasite digest ample quantities of haemoglobin (between 30 and 70%) to release haem⁽²⁴⁾. Since haem is toxic to parasites, majority of haem is changed to crystalline haemozoin, the malaria pigment⁽⁶⁾. The extent of haemoglobin degraded is dependent on the stages of parasite infection, as haem crystallization is lower in early-stage parasites (rings) and increases as parasites mature through the mid (trophozoite) and late (schizont) stages⁽²⁴⁾. The process of haem crystallization is the target for antimalarial drug, chloroquine, which binds to haem and induces an increase in labile haem⁽⁶⁾. Erythrocyte deformity leads to a loss of shape, increased membrane rigidity, permeability and adhesiveness to vascular endothelium (cytoadherence) and non-infected erythrocytes (rosetting)^(25,26). Erythrocyte deformity enables parasites survival within host cell and increase disease virulence that include cerebral malaria and anaemia⁽²⁶⁾. Sequestration of infected deformed adhesive erythrocytes to the brain is the primary cause of coma manifested in cerebral malaria⁽²⁷⁾. The reason for infected erythrocyte sequestration is unknown, but it is speculated that parasites develop better in an oxygen-depleted environment and endothelium binding helps parasites escape spleen-dependent destruction⁽²⁵⁾.

Therefore, antimalarials directed at preventing *Plasmodium* invasion into erythrocytes, thereby stabilizing the erythrocyte membrane, could be considered as antimalarial agents. However, antimalarial drugs such as artesunate, mefloquine, and quinine are believed to exert their antimalarial effects by destabilizing the erythrocyte membrane, thus causing eryptosis^(28,29). Evidence of erythrocyte membrane destabilization by *Plasmodium* parasites, leading to anaemia, increased haemozoin and haem concentrations, which were confirmed

in our study. Our present study also shows that alkaloids of *Phyllanthus amarus* has positive effects against *Plasmodium* infection. *P. amarus* directly targets parasites by inhibiting erythrocytes invasion by stabilizing erythrocyte membrane. This was accompanied by reduction in parasitaemia, an attenuated parasite clearance and improvement of haematological parameters in experimental mice. Chopade *et al*⁽¹²⁾, demonstrated that the anti-inflammatory activity of *P. amarus* is due to its membrane stabilizing action and inhibition of protein denaturation, as aqueous extract of *P. amarus* inhibited erythrocyte haemolysis and protein denaturation.

Haematological parameters are often needed to assist in the management of malaria infection⁽³⁰⁾. Low platelet count, decreased PCV and Hb concentrations have been reported to be a strong indicator of malaria⁽³¹⁾. Therefore, in malaria endemic areas, patients manifesting these symptoms should be further tested for malaria. Decreased PCV and RBC is associated with increased haemolysis of infected and non-infected red blood cells in malaria infection⁽³²⁾. Non-infected RBCs have been reported to have a shorter life span in comparison with non infected RBCs in healthy individuals, with their haemolysis accounting for a greater loss (over 90%) in acute anaemia⁽²⁾. Iron sequestration and deficiency also implicated in malaria can contribute to anaemia⁽³³⁾. Impaired erythropoiesis has also been reported^(34,35). Impaired erythropoiesis has been related to increased production of proinflammatory cytokines, produced in response to malaria (eg. TNF- α), which inhibits all stages of erythropoiesis⁽³⁶⁾. Also, large haemozoin deposits found in bone marrow of children that died from severe malaria, suggests a direct inhibition of erythropoiesis by haemozoin⁽³⁷⁾. Haemozoin has also been associated with direct inhibition of reticulocyte formation⁽³⁸⁾. Anaemia is also induced in malaria via CD8⁺ T cell-dependent plasmodial clearance and red cells removal in the spleen⁽³⁹⁾. It may also be due to normocytic, normochromic anaemia (anaemia of chronic disease) seen in malaria infected subjects⁽³²⁾.

Increased concentration of haem, as reported in our study has also been confirmed by other researchers^(40,41). Free haem released during haemoglobin digestion in erythrocytes, leads to increased concentration of haem as not all haem undergoes conversion⁽¹⁾. At the same time, the parasites possess a haem *de novo* biosynthetic pathway⁽⁴⁰⁾, which leads to an increased concentration of haem. Also, increased haemolysis of red cells leads to the release of free haem. Free haem released during haemoglobin digestion and haemolysis converted to haemozoin⁽⁴²⁾ causes an increased concentration of haemozoin which is also reported in our study. A positive correlation between haemozoin concentrations and peripheral parasitaemia has been reported⁽⁴³⁾. Lethal forms of *Plasmodium*, like *Plasmodium falciparum*, presents with a higher concentration of haemozoin in comparison with milder forms of *Plasmodium* even at same level of parasitaemia⁽⁴³⁾. While free haem is toxic to parasites, haemozoin disrupts hosts normal function. This is said to be due to the production of lipoperoxides from arachidonic acid by haemozoin⁽⁴⁴⁾. One could, therefore, speculate that the *de novo* biosynthetic pathway and the haem detoxification pathway be targeted for antimalarial drug discovery. Ke *et al*⁽⁴⁰⁾ demonstrated that the haem biosynthetic pathway is not essential for asexual blood-stage growth of *P. falciparum* parasites, but is required for mosquito transmission, hence are not good targets for antimalarial drugs. Nevertheless, Chandana *et al*⁽⁴¹⁾, showed that griseofluvin,

targeted at inhibiting parasites haem biosynthesis, reduced the risk of cerebral malaria. However, drugs that inhibit haem detoxification to haemozoin have proven to decrease parasitaemia. 3-hydroxy-11-keto- β -boswellic acid have been found to inhibit haem detoxification pathways and increase survival time in *P. yoelii nigeriensis* infected mice⁽⁴⁵⁾. Chloroquine's primary mechanism of action involves disruption of haemozoin formation⁽⁵⁾. Other antimalarials (quinine and artemisinin) have also been reported to block the formation of haemozoin⁽⁴⁾. Haemozoin inhibitors are effective as they cause a dose-dependent increase in exchangeable haem, and correlates with decreased parasite survival, as free haem is toxic to parasites. Electron spectroscopic imaging showed that haemozoin inhibitors work by relocating of haem iron into the parasite cytoplasm, while electron microscopy provided evidence of the disruption of haemozoin crystals⁽⁴⁶⁾.

Mild thrombocytopenia, characterized with low platelets counts are common in malaria infected patients^(30,32). This is also confirmed by our study. The low platelet count has been reported to be the strongest predictor of malaria, with spontaneous recovery on treatment⁽³¹⁾. Mild splenomegaly reported in some malaria infected patients might be the cause of thrombocytopenia⁽³²⁾. Also, it has been reported that infected red cells become adhesive for platelets, endothelial cells and uninfected erythrocytes⁽⁴⁷⁾. Erythrocyte adhesiveness has been linked with lysis of red cells⁽²⁾ and damage to endothelium⁽⁴⁸⁾. Therefore, the platelets face the same fate as red cells and endothelium, hence reduced platelets count. Other studies suggest that the mechanisms for thrombocytopenia include disseminated intravascular coagulation, or excessive removal of platelets by reticulo-endothelial system⁽⁴⁹⁾. Thrombocytopenic malaria, in contrast to the non-thrombocytopenic variety correlates with a higher degree of parasitaemia and increased cytokine production^(50,51). Hence, thrombocytopenia is positively correlated with parasite density.

On the other hand, white cell count is not useful to predict *falciparum malaria*^(31,52). Some studies have reported increased concentrations (leukocytosis)⁽⁵³⁾ while others have revealed reduced concentrations of white cells (leucopenia) following malaria infection^(31,54) and other studies reveal no significant change in concentrations⁽⁵²⁾. Our study reports leucopenia in *P. berghei* infected mice. Neutrophil and lymphocyte counts have been, however, reported to be the most important leukocytic changes associated with the *Plasmodium* infection⁽⁵⁵⁾. Reduced neutrophil⁽⁵³⁻⁵⁶⁾ and lymphocyte⁽⁵⁴⁻⁵⁷⁾ in *Plasmodium* infection has been reported. The decrease in lymphocyte counts associated with malaria as observed in this study may be due to reflection in redistribution of lymphocytes with sequestration in the spleen⁽⁵⁵⁾. Neutrophil count have been reported to be increased in patients with higher levels of parasitaemia than patients with low and moderate parasitaemia⁽⁵⁴⁾. This suggests that neutrophil concentrations shows a positive correlation with parasite density. Low eosinophil concentrations have also been reported⁽⁵⁴⁾.

Conclusion

Our study demonstrated that *P. berghei* (NK65) parasite induced malaria in experimental mice (albino BALB/c strain) with associated anaemia, erythrocyte membrane destabilisation and increased serum haemozoin content and haem

level. However, treatment of the malarial infection with alkaloid extract of *P. amarus* significantly cleared blood parasite with improvement in the anaemic condition and erythrocyte destability status in a dose-dependent manner and in trends that compared well with the standard drug treatment. The alkaloid extract of *P. amarus*, also further increased serum haem concentrations while reducing haemozoin content. Alkaloid extract of *P. amarus* has the potential to clear blood malarial parasite, stabilize the membrane of *Plasmodium*-damaged erythrocytes and further increase haem content. Therefore, one of the mechanisms of *P. amarus* alkaloid extract as an antimalarial is by inhibiting the process of polymerizing haem to soluble, non-harmful, malarial pigment; haemozoin. So, alkaloids of *P. amarus* can be critical in development of new and more effective antimalarial agents and this should be rigorously pursued.

Ethical Approval

This study was approved by the Research and Bioethics Committee, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

Competing interests

The authors declare that there are no conflict of interests.

Authors' Contributions

Onyesom I, designed, supervised the research and vetted the draft manuscript for approval and submission. Opajobi AO, Ojugbeli ET, Uzuegbu UE, Toloyai PY and Oshiegbu W, conducted the laboratory experiments and design, while, Elu CO, analyzed, interpreted the data and prepared draft manuscript which was eventually approved by all authors for submission

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