Evaluation of erythrocyte viability, antioxidant capacity and antiplasmodial activity induced by alkaloid extract of *Phyllanthus amarus*

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ABSTRACT

Introduction: The production of large amounts of reactive oxygen species in severe malarial infection is due to parasite invasion to erythrocytes. Malaria resistance to medication has left malaria-endemic countries with no alternate source of medications but traditional medicine. One of such plants utilized by traditional healers is *Phyllanthus amarus*. Therefore, this study aims at ascertaining the antiplasmodial and cytotoxic activities of *P. amarus* and its specific actions on retaining erythrocyte viability and antioxidant activity.

Methods: Antiplasmodial and erythrocyte viability activities were determined *in vitro* via parasite suppression and tetrazolium-based colorimetric assays, respectively. Antioxidant capacity was determined by measuring extract's ability to inhibit lipid peroxidation, scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide ions, reduce iron(III) ions, and chelate iron *in vitro* using documented methods.

Results: Alkaloid extracts of *P. amarus* showed great antiplasmodial activity (IC_{50}=0.52μg/mL) and low cytotoxic activity (CC_{50}=54.95 μg/mL). Erythrocyte viability assay showed the minimal impact of the extract on the uninfected erythrocytes but improved viability of the infected RBC in a dose dependent manner, and antioxidant activity manifested mainly in its iron chelating activity (EC_{50}=0.34 μg/mL).

Conclusion: This study suggests that the alkaloid extract of *P. amarus* has significant antiplasmodial and antioxidant activities. These activities promoted the repair of parasite-induced free radical damage to the erythrocyte membrane but distorted the parasites redox balance and defense mechanism, and hence survival rate as indicated by the parasite suppression associated with alkaloid extract treatment of malarial infection.

Implication for health policy/practice/research/medical education:
The demonstrated antiplasmodial and antioxidant activities of the alkaloid extract of *P. amarus* significantly repaired the membranes of parasite-damaged erythrocytes. Therefore, the plant's alkaloid phytochemical is non-toxic, unlike several other natural and synthetic alkaloid-based antimalarials. This should be taken into consideration in subsequent studies on malaria therapies involving alkaloids of *P. amarus*.

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Introduction

The burden of malaria makes the disease remain one of the most important parasitic diseases in Africa (1). The struggle associated with its elimination has been linked to malaria resistance to treatments. The development of resistant strains of the malarial parasite results from constant changes in the genetic makeup of the parasite (2).

Hence, the constant search for alternative, more efficient, and affordable medications is needed.

The pathogenesis of malaria is associated with the generation of large amounts of reactive oxygen species (ROS), leading to oxidative stress (3). ROS production is as a result of haemoglobin degradation by parasites, which produces free heme and hydrogen peroxide

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(H_2O_2) (4). Oxidative stress, if left untreated, leads to complications, including anaemia, tissue damage, and cerebral malaria, commonly seen in severe manifestations of the infection (5). The metabolic pathways associated with the production of ROS in malaria infection can, therefore, serve as targets for antimalarial drug discovery. Antioxidant compounds are important in neutralizing harmful free radicals in the body. Antioxidant compounds could be free radial scavengers or reducing agents (6). So, searching for medicinal plants with antioxidant and antiplasmodial activities could lead to the development of new antimalarials.

Phyllanthus amarus has been utilized traditionally for its antidiabetic, antihypertensive, hepatoprotective, analgesic, antimicrobial, anti-inflammatory, anticarcinogenic, antimutagenic, and antimalarial properties as documented by researchers (7-9). Sequel, the antiplasmodial activity of *P. amarus,* has been consistently confirmed (10-12). The plant has also been observed to invigorate the blood (13) and protect renal (14,15), testicular, and ovarian (16) functions from an oxidative assault during malarial infection. Alkaloid extract of *P. amarus* has been reported (17,18) to be the main phytochemicals responsible for the observed antiplasmodial activity of the plant, but it's antioxidant activity and impact on damaged erythrocytes are yet to be known. In this study, therefore, we investigated the antioxidant potential (scavenging, reducing power, chelating activity, and peroxide inhibition) of *P. amarus* alkaloid extract and its impact on the viability of both normal and parasite-damaged red blood cells (RBCs), having confirmed the antiplasmodial activity and cytotoxicity of the phytochemical extract.

Material and Methods
Collection and extraction of plant materials
The whole plant of *P. amarus* was collected from farmlands in Abraka community, Ethiope East Local Government Area of Delta State, Nigeria, and identified at the Forestry Research Institute of Nigeria, Ibadan, Oyo State, Nigeria. A specimen with herbarium number FHI 109728 was deposited there. Leaves were plucked, washed and air dried at laboratory room temperature (28°C-32°C). After two weeks, the dried leaves were pulverized using a laboratory blender (Kenwood, Japan). Alkaloid extract was obtained according to the method stated by Uzuegbu et al (18). About 100 g of the powdered leaves were mixed in ammonia (100 mL for 1 hour) and thereafter dichloromethane (250 mL for 24 hours), filtered, and the residue was added to H_2O/HCl and filtered. The resulting filtrate was again treated several times with dichloromethane (6 mL × 150 mL). The extracting solvent was then evaporated and the residue was concentrated to dryness under residual pressure. The extracts were confirmed with Dragendorff reagent, and the dilutions were prepared to obtain the final concentrations of extracts used in the study (18).

Antiplasmodial assay
The strain of *P. falciparum* (Chloroquine sensitive *P. falciparum* 3D7) was maintained at 5% hematocrit (human type O-positive RBCs) in complete RPMI 1640 (supplemented with 10% Albumax II, 50 μg/mL gentamycin, 1% L-glutamine) medium. Antiplasmodial activity (the median inhibition concentration, IC_{50}) was determined by the parasite growth inhibition assay (18).

Cytotoxicity assay
Cytotoxicity (median cytotoxic concentration, CC_{50}) of the alkaloid extract was assessed against animal cell fibroblast L929 cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 0.21% sodium bicarbonate and 5 mg/mL gentamicin at 37°C using the DMSO-LDH method (18).

Erythrocyte viability assay
Tetrazolium-based colorimetric assay was used to assess erythrocyte viability (19). About 100 μL of varying concentrations of the extract and 100 μL of *P. falciparum*-infected RBCs (1.5% parasitaemia) were added to wells of 96-well plates. Uninfected erythrocytes in media served as the positive control. Assay was performed in triplicates. Wells were incubated at 37°C for 5 days at 5% O_2 and CO_2. Then, 20 μL of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well and incubated under the same conditions for 2 h. Exactly, 150 μL of culture media were removed from each well and discarded and 200 μL of Triton X-100 in acidified isopropanol were added to each well. The plates were then kept at room temperature in the dark for 24 hours, and the optical densities of the wells were read at 565 and 690 nm on a spectrophotometer (Spec 20D, Techmel and Techmel, USA). Erythrocyte viability was then calculated with the following formula:

Optical density = [(Blank absorbance – Sample absorbance)/ Blank absorbance × 100]

Lipid peroxidation assay
Lipid peroxidation was determined according to the methods described by Ohkawa et al (20). *P. amarus* alkaloid leaf extract (ranging between 10-100 000 μg/mL) was mixed with the media (250 μL of egg yolk homogenate) and the volume was adjusted to 500 μL with distilled water. Then, 25 μL of ferrous sulphate was added to the mixture and incubated at 25°C for 30 minutes. Thereafter, 750 μL of acetate (pH 3.4), 750 μL of thiobarbituric acid and 25 μL of trichloroacetate were added to the reaction mixture and incubated further for 60 minutes in boiling water. Three ml of 1-butanol was added to the mixture after cooling and centrifuged at 940 × g for 10 minutes. The absorbance of the upper layer was measured at 532 nm with a spectrophotometer (Spec 20D, Techmel and Techmel, USA). Lipid peroxidation inhibition activity was

evaluated using dose-response curves.

Free radical scavenging assay

Assay was carried out using the method described by Govindarajan et al (21) with some modifications. Scavenging activity of *P. amarus* was based on the scavenging activity of the 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical. Alkaloid extract (15.6 - 500 µg/mL) and the standard (ascorbate) were dissolved in methanol and centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected. Then, 1 mL of the supernatant was added to 3 mL of DPPH, dissolved in methanol. Absorbance at 517 nm was determined after 30 minutes. Thereafter, 1 mL of methanol was added to 3 mL DPPH solution, incubated at 25°C for 1 hour and used as control. Methanol was used as the blank. Percentage inhibition activity was calculated from the following formula:

DPPH scavenging activity (%) = $[(A_0 - A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

The results were expressed as percentage DPPH scavenging effect against log concentration, and the EC_{50} was determined.

Ferric iron reducing power assay

The reducing capacity of extract (0.1-3.0 mg/mL) was determined using the method of iron(III) ions, Fe^{3+}, to iron(II) ions, Fe^{2+} described by Santhosh Kumar et al (22). About 1 mL of the extract was mixed with 2.5 mL of phosphate buffer (pH 6.5) and 2.5 mL of potassium ferricyanide solution. The mixture was incubated at 50°C for 20 minutes. Trichloroacetate solution (1.5 mL, 10%) was added to the mixture and centrifuged at 3000 rpm for 10 minutes. 2.5 mL of supernatant was taken from the mixture and added to 0.5 mL of 0.1 % ferric chloride solution and made to volume with 2.5 mL of distilled water. Absorbance was measured at 700 nm. Distilled water was used in place of extract for the blank. The percentage reduction was calculated using the following formula:

Ferric iron reducing capacity (%) = $[(A_0 - A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance of alkaloid extract or the standard sample.

Ion (Fe²⁺) chelating assay

Metal chelating activity was measured by the method described by Ahmed et al (23). About 0.1 mM of ferrous sulphate ($FeSO_4$) was added to 0.25 mM of ferrozine to form a Fe^{2+}-ferrozine complex. Varying concentrations of the extract were added to the complex and incubated at room temperature (20-25°C) for 10 minutes. Absorbance was measured at 562 nm. Ethylene diamine tetra acetic acid (EDTA) was used as a positive control. Percentage chelation was calculated using the following formula:

Chelating activity (%) = $[(A_0 - A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance of alkaloid extract or the standard sample.

Superoxide scavenging activity

The superoxide anion scavenging activity was measured based on the already described method (24). Superoxide radicals were generated in a phenazine methosulfate-nicotinamide adenine dinucleotide-nitroblue tetrazolium chloride (PMS-NADH-NBT) system. The system consisted of 3 ml of sodium phosphate buffer (pH 7.4), 1 mL of NBT solution, 1 mL of NADH solution, and different concentrations of the alkaloid extract (25–250 µg/mL) in water. About 1 mL of PMS solution was added to the mixture. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance was measured against the corresponding blank solution. Ascorbate was used as the positive control. The percentage of superoxide radical scavenging was calculated using the following formula:

Superoxide scavenging activity (%) = $[(A_0 - A_1)/A_0 \times 100]$

where A_0 is the absorbance of the control and A_1 is the absorbance of alkaloid extract or the standard sample.

Statistical analysis

Data were analyzed with the analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software Inc, LLC, San Diego CA, USA, version 6). Results were expressed as mean ± SD and were used in plots of activity of the extract against the log of extract concentrations to determine concentrations at 50% activity.

Results

The results obtained from the present investigation into the antioxidant potential of *P. amarus* alkaloid extract and its associated impact on erythrocyte viability and antiplasmodial activity are given in Figures 1-4 and Table 1 as follows.

Antiplasmodial and cytotoxic activity

The *in vitro* antiplasmodial and cytotoxic activities of *P. amarus* alkaloid leaf extract was evaluated in this study by assessing the antimalarial activity of varying concentrations of extract and chloroquine (CQ) against *P. falciparum* in culture. Parasite suppression of the extract and standard drug (CQ) at different concentrations are shown in Figure 1.

There was a dose-dependent increase in parasite suppression by the alkaloid leaf extract comparable to the

antiplasmodial activity of the standard drug. Based on the categorization of antiplasmodial activity by Uzuegbu et al (18) IC$_{50}$ of extract (0.52 µg/mL) was less than 5 µg/mL, which makes it a very potent antiplasmodial compound.

Cytotoxic activity of the extracts and standard drug at different concentrations are illustrated in Figure 2.

There was also a dose-dependent increase in cytotoxicity induced by the alkaloid leaf extract, which was comparable with the standard drug. The alkaloid extract was non-toxic (CC$_{50}$ = 54.95; >30 µg/mL). Alkaloid extract of *P. amarus* was, therefore, highly selectable as an antiplasmodial agent (Selectivity index, SI = CC$_{50}$/IC$_{50}$, was 105.67), since the index was greater than 10 (18).

Erythrocyte viability

Results from the erythrocyte viability assay are illustrated in Figure 3. Erythrocyte cell membrane integrity of parasitized erythrocytes was significantly improved in a
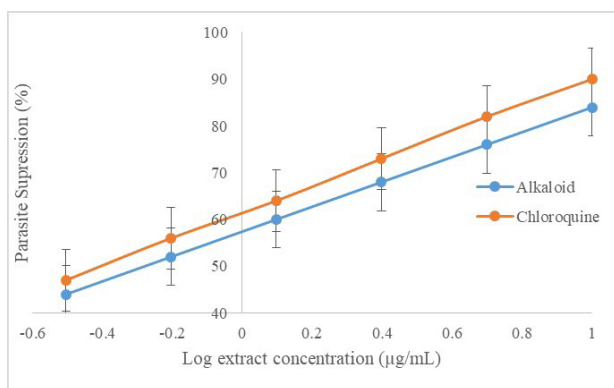

Figure 1. Antiplasmodial activity of alkaloid extract of *Phyllanthus amarus*. IC$_{50}$ of alkaloid and chloroquine are 0.52 µg/mL and 0.41 µg/mL, respectively. IC$_{50}$ = Inhibition concentration at 50% *P. falciparum* suppression, IC$_{50}$>10 µg/mL is inactive; >5–10 µg/mL is moderately active; ≤5 µg/mL is active (18).

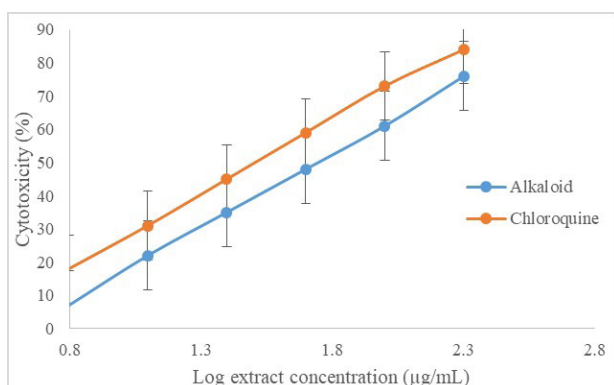**Figure 2**. Cytotoxicity of alkaloid extract of *Phyllanthus amarus*. CC$_{50}$ values of alkaloid and chloroquine are 54.95 µg/mL and 31.62 µg/mL, respectively. CC$_{50}$=Cellular cytotoxic concentration at 50% toxicity, CC$_{50}$<1 µg/mL is highly toxic; 1–10 µg/mL is moderately toxic; 10–30 µg/mL is mildly toxic and >30 µg/mL is non-toxic (18).

dose-dependent manner, but the impact of the alkaloid extract on the uninfected erythrocytes was minimal, confirming the non-toxicity of the extract.

Antioxidant activity

Antioxidant capacity of the alkaloid extract was explored using lipid peroxidation inhibition, free radical scavenging, ferric iron-reducing power, iron chelating, and superoxide radical scavenging activity. There was a dose-dependent increase in antioxidant activity in all instances (Figure 4). However, judging by the results is presented in Table 1; the antioxidant activity of the alkaloid extract of *P. amarus* seems to reside in its metal chelation ability.

Discussion

RBC invasion by *Plasmodium* parasite produces a host of destructive changes, including the loss of shape, rigidity of membrane, increased permeability, and increased adhesiveness of red cells (25). Malaria chemotherapy directed as sustaining the viability and/or protecting erythrocytes from assault should provide promising antimalarial drug development tactics as RBC protection would immediately stop parasite multiplication in the host (26). Therefore, exploring the activity of traditional plants with reputable antiplasmodial activity could provide alternate therapy. The antiplasmodial activity of the alkaloid leaf extract of *P. amarus* in this study showed that the extract possesses significant antiplasmodial activity with no toxicity on animal cell fibroblasts *in vitro*. This is in consonance with the studies of other researchers. The antiplasmodial activity (10-12,15-18) and the zero to minimal toxicity of *P. amarus* (12,18,19) have been reported.

Erythrocyte viability assay showed that the treatment of infected erythrocytes with alkaloid extract of *P. amarus* increased viability by 27% at the highest concentration. Uninfected erythrocyte cultured with the same extract showed a 3% increase in viability at the lowest concentration and 4% and 2% decrease in viability at 300 and 500 µg/mL, respectively, when compared with untreated erythrocytes. This suggests that administration of alkaloid extract to *P.*
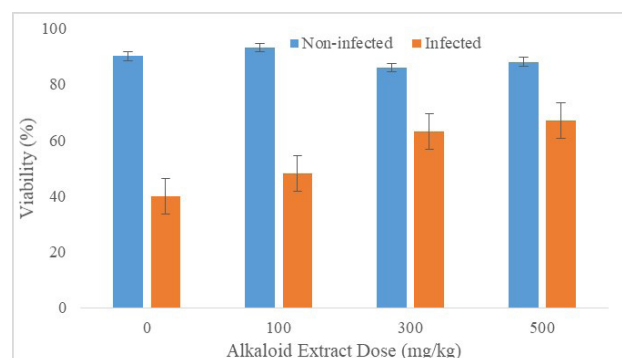

Figure 3. Erythrocyte viability induced by alkaloid extract of *Phyllanthus amarus*.
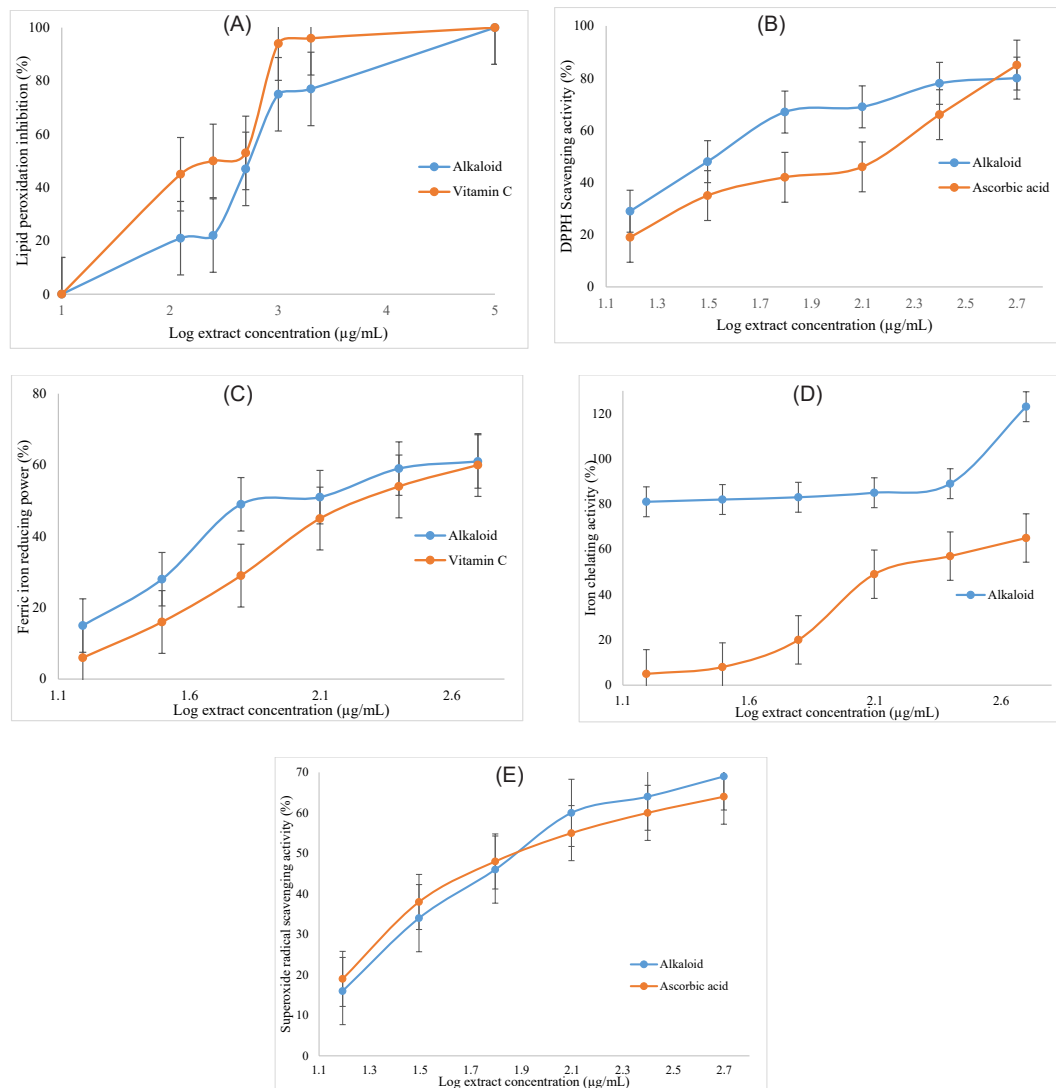
Figure 4: Antioxidant activity of alkaloid extract of *Phyllanthus amarus*. A= inhibition of lipid peroxidation activity, B= Free radical (DPPH) scavenging activity, C= Ferric iron-reducing activity, D= Iron (Fe^{2+}) chelating activity, E= Superoxide radical scavenging activity.

falciparum-infected erythrocytes significantly increases the viability of RBCs. This could be due to the protection of RBCs membranes from parasite invasion or reversing the effects of the parasite. Although, there is no vast available study on the erythrocyte protective mechanism of *P. amarus,* Olawale et al (27) reported that the aqueous extract of *P. niruri* protects against severe malaria by protecting the erythrocyte membrane from parasite invasion.

Erythrocyte membrane lipids are susceptible to attack and oxidation by ROS to form lipid peroxides (28). It has been established that increased erythrocyte lipid peroxidation following malaria infection is accompanied by decreased antioxidant status (4). Therefore, *Plasmodial* phospholipid synthesis inhibitors could be considered potential antimalarial agents. Results from this study suggest that the alkaloid extract of *P. amarus* inhibited lipid peroxidation to a substantial amount but not as great as vitamin C. However, at higher concentrations (100 000 µg/mL), the inhibition of lipid peroxidation was

Table 1. Concentrations at 50% antioxidant activity

	Lipid peroxidation inhibition	DPPH scavenging activity	Fe^{3+} reducing activity	Fe^{2+} chelation	O^{2-} scavenging activity
Extract	537.03	33.88	63.10	0.34	74.13
Standard	251.19	144.54	173.78	125.89	75.86

All values are concentrations (µg/mL) recorded at 50% activity. Fe^{3+}= iron(III) ions, Fe^{2+}= iron(II) ions, O^{2-}= Superoxide ions, DPPH= 1,1-diphenyl-2-picrylhydrazyl.

maximum, even at the same level of vitamin C (100%). This may suggest that higher concentrations of the extract are needed for treatments. Also, other studies have reported the ability of *P. amarus* extracts to inhibit lipid peroxidation. Mallaiah et al (29) and Ogunmoyole et al (30) have reported the inhibitory lipid peroxidation effects of the extracts of *P. amarus* in patients infected with malaria. Londhe et al (31) showed that the polyphenol constituents of *P. amarus* protect rat liver mitochondria membrane against oxidative damage. Further confirming this activity, Nikam et al (32) also reported that the extracts of *P. amarus* inhibit erythrocyte lipid peroxidation in ethanol-induced liver damage and associated this activity with polyphenol constituents of *P. amarus*.

The efficacies of antioxidants are usually associated with their ability to inhibit oxidative damage by scavenging free radicals. Plants with high phenolic content are great antioxidants as the antioxidant activity of plant extracts correlates with the content of their phenolic compounds (33). Some free radical scavengers have been studied for their antiplasmodial activities. Reis et al (34) study on antioxidant therapy with N-acetyl cysteine and deferoxamine as additives to chloroquine prevented insistent cognitive damage in cerebral malaria. The results of this study show that the alkaloid extract of *P. amarus* possesses great free radical scavenging activity, even greater than the standard drug, ascorbate. The results from our study are comparable with the standard drug and results from other studies. Mallaiah et al (29) and Ujah et al (35) reported the great free radical scavenging ability of *P. amarus* extracts, and this activity had been attributed to the high phenolic constituents of the plant.

A compound's reducing power is dependent on its ability to transfer electrons, and this function is an indicator of its antioxidant activity (36). Therefore, they transform free radicals into less reactive species by eliminating free radical intermediates, inhibiting further oxidation. Antioxidant molecules induce the reduction of Fe^{3+}/ferricyanide complex to its ferrous (Fe^{2+}/ferricyanide) form due to their reducing potential (37). Our study shows that the alkaloid extract of *P. amarus* has significant reducing power. The iron-reducing power of *P. amarus* has been reported by Dinesha et al (38).

Antioxidants that can be explored in malaria therapy include iron chelators that bind to toxic metal ions to form easily excreted compounds (39). *Plasmodium* parasites require iron for their reproduction; therefore, drugs withholding available iron could halt its reproduction. A major characteristic of anaemia, which is also manifested in severe malaria, is iron overload (40). Iron generated from anaemia can accelerate the formation of free radicals by oxidation or reduction (41). It is also important to note that chelation therapy has also been employed in neutralizing iron overload in anaemia (40). Therefore, chelation of iron can reduce the formation of free radicals in severe malaria. Iron chelating drugs, such as

deferiprone, deferoxamine, and deferasirox, the common iron chelators employed in disorders presenting oxidative stress, have also been shown to demonstrate both *in vivo* and *in vitro* antimalarial effects (42). Our study highlights that the alkaloid extract of *P. amarus* also possesses great iron chelating activity when compared with the standard, sodium ascorbate. The results also highlight that the antioxidant activity of the extract of *P. amarus* resides in iron chelation. This is in agreement with the report by Dinesha et al (38), who demonstrated that polysaccharides from *P. amarus* possess great iron chelating activity.

Free radicals produced in oxidative stress include; superoxide ions (O_2^-), hydroxyl peroxide (H_2O_2), and hydroxyl radicals (OH^-). Enzymatic antioxidant defense mechanisms are present in the body to counter oxidative stress (41). An example of such an enzyme is the superoxide dismutase, which is involved in the dismutation of O_2^- to H_2O_2 and is reduced by other enzymes to water, preventing its reaction with iron. However, in severe malaria, there is a redox imbalance between ROS generation and neutralization by the enzyme antioxidant system (4). This also highlights the importance of antioxidant medication in malaria therapy. Ebohon et al (43) showed that antimalarial drug, artesunate–amodiaquine in combination with vitamin C, increased superoxide dismutase activity and reduced hydrogen peroxide and malondialdehyde levels in *P. berghei*-infected mice when compared with the mice treated with only artesunate-amodiaquine in hepatic and renal tissues. From our study, *P. amarus* alkaloid extract demonstrated significant superoxide scavenging activity. Superoxide scavenging activity of alkaloid extract could be as direct neutralization of superoxide radicals or by increasing the activity of the superoxide dismutase.

Conclusion

Based on current observations, the alkaloid extract of *P. amarus* demonstrated significant antiplasmodial activity with great antioxidant potential, particularly iron chelating ability, which may have supported the repair of parasite-damaged erythrocytes and hence, improvement in their viability. The active alkaloid compounds, which possess antiplasmodial and antioxidant (iron chelating and scavenging) activities, should be identified for further study.

Authors' contributions

OI conceived and designed the study. He also supervised the research at every stage and vetted the draft manuscript for approval and submission. UUE and OAO conducted

the laboratory experiments as designed, while ECO wrote the draft, performed the revision of the manuscript and data analysis, which was eventually approved by all authors for submission.

Conflict of interests

The authors declare that there is no conflict of interest to declare.

Ethical considerations

All ethical issues including (etiquette and ethics, authorship issues, data fabrication, duplicate publication, falsification, and plagiarism) have been carefully observed by all authors.

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