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PROFILING OF *PHYLLANTHUS AMARUS* PHYTOCHEMICAL CONSTITUENTS AND EVALUATION OF ASSOCIATED ANTIMALARIAL ACTIVITY AND ANTIOXIDANT POTENTIAL IN EXPERIMENTAL MICE

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Abstract
The failing curative ability of antimalarials has prompted an open discussion for the use of antioxidants in combination with antimalarials for effective chemotherapy. This study profiled the phytochemical constituents of Phyllanthus amarus and evaluated their antimalarial and antioxidant activities. Phytochemical screening, antimalarial and acute oral toxicity were determined according to standard procedures. Blood antioxidant activity was assessed by measuring antioxidant enzymes and nitric oxide (NO), concentrations of malarial infected mice treated with P. amarus phytochemicals. Alkaloids, the most abundant phytochemical, demonstrated the highest malarial parasite chemosuppression and greatly improved NO concentration in infected mice. However, flavonoid extract demonstrated the highest antioxidant potential with most impact on catalase activity and malondialdehyde concentration. Alkaloid may function as antimalarial agent by inhibiting haem polymerization to hemozoin as judged by its increased effect on NO concentration which bears an reverse relationship with hemozoin.

Keywords: *Biomarkers, Nitric Oxide, Haemolysis, Oxidative Stress, Hemozoin, Malaria*

INTRODUCTION

Malaria, a highly oxidative disease, is an important public health problem causing an estimated 500 thousand deaths yearly, with majority of these deaths occurring in the African region. The 2021 World Malaria Report, recorded an estimated 241 million malaria cases and 627,000 global deaths in 2020, which is a drastic increase in cases (14 million more cases) in comparison with 2019. Increased cases has been linked to the COVID-19 pandemic disruptions in malaria prevention, diagnosis and treatment in 2020[1].

Malaria, being an oxidative disease, induces oxidative stress, a condition that results in increased rate of cell damage. Malaria has been reported to cause oxidative stress, leading to anaemia, cerebral malaria and even death in severe cases[2]. Although, some malaria medication are said to be effective due to their pro-oxidant effect[3], the use of antioxidants in malaria chemotherapy to counteract oxidative stress cannot be neglected. Antioxidants; N-acetyl cysteine and desferroxamine have been shown to be very effective in combination with antimalarial medications[4]. It has been reported that the use of antioxidants as adjuvants in malaria therapy, reduces the risk of complications which could otherwise lead to severe malaria[5].

Medicinal plants have been shown to have antimalarial activity. Some owing to their antioxidant activity, while others, because of their direct action on *Plasmodium* parasitic stages in blood[6]. *Phyllanthus* (*P.*) *amarus*, the plant investigated in this study, has been used by traditional healers in Nigeria to treat malaria and other inflammatory ailments[7].

Therefore, in this study, we profiled the phytochemical constituents of *P. amarus* and assessed their antiplasmodial and antioxidant activities in experimental mice.

Experimental

Collection of Plant Materials and Preparation of Phytochemical Extracts

Fully developed whole plants of *Phyllanthus amarus* were collected from farmlands in Abraka community located in Ethiope East Local Government Area of Delta State, Nigeria. Plants were identified at the Forestry Research Institute of Nigeria, Ibadan, Oyo State, Nigeria. Plants were then, washed and air dried at room temperature (28 ^{o}C - 33 ^{o}C) to constant weight and thereafter ground to powder using laboratory blender (Kenwood, Japan).

The phytochemicals; alkaloid[8], tannin[9], flavonoid[10], saponin[11], anthraquinone[12] and glycoside[13] were identified and extracts were prepared by standard methods (already described).

Experimental Animals

Eight weeks old Swiss mice (BALB/c albino strain; 24 – 28 g bwt.) were obtained from the Laboratory Animal Centre, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria. The experimental mice were maintained in strict compliance with guidelines approved by the Research and Bioethics Committee, Faculty of Basic Medical Sciences, Delta State University, Abraka, Nigeria.

Acute Oral Toxicity

Animals were separated into control groups (positive and negative control groups) and six test groups consisting of five mice for individual doses of 10, 100, 1000, 2000, 3000 and 5000 mg/kg. Acute oral toxicity was determined according to methods previously described by the OECD (Organization for Economic Co-operation and Development).

Parasites and Inoculation

Donor mice already infected with *P. berghei* parasites (Strain NK65), were procured from the Department of Parasitology, Nigerian Institute of Medical Research, NIMR, Yaba, Lagos State, Nigeria. The experimental mice were infected by obtaining parasitized blood from the cut tail tip of the infected (donor) mice. The inoculum was prepared by diluting 0.1 mL of infected blood in 0.9 mL of phosphate buffered saline (pH 7.2)

Antiplasmodial Activity of Phytochemicals

Antiplasmodial activity in established infection was evaluated using methods previously described by Onyesom et al[14]. Seventy-two hours (72 h) after inoculation, blood smears were made to determine parasitaemia and confirm success of inoculation before treatment commenced. Mice were thereafter separated into twenty (20) groups of five, based off mean parasitaemia. Groups treated with standard drug (Lonart®DS) and distilled water, served as standard and negative controls, respectively. Test groups consisted of five mice for individual doses of 100, 300 and 500 mg/kg for each phytochemical. Parasitaemia was determined at Day 0, 3, 6, 9 and 12 by thick blood smears made by collecting blood from the cut tail tip of only the infected mice and stained with Giemsa stain which was later viewed under the microscope (TH- 9845, Serico, China) at ×40 magnification. Percentage parasitaemia and suppression were calculated with the following formulae:

% Parasitaemia = (Number of parasitized RBCs / Total number of RBCs) ×100

% Chemosuppression = [(Mean parasitaemia of negative control – Mean parasitaemia of treated group) / Mean parasitaemia of negative control) × 100

Determination of Haematological Indices
Blood samples collected were used for haematological analysis. Packed cell volume (PCV), red blood cell (RBC), haemoglobin (Hb), white blood cells (WBC), platelet count and differential white blood cells (monocytes, neutrophils) were determined using an hematology analyzer (Human Diagnostics Human Count, 80).

Antioxidant Activity Assays
Blood was allowed to clot and serum was separated with pipettes into other plain bottles and stored for analysis.

Antioxidant enzymes; catalase, CAT[15], glutathione peroxidase, GPx[16], glutathione S-transferase, GST[16], superoxide dismutase, SOD[17], Myeloperoxidase, MPO[18] activities and concentrations of reduced glutathione, GSH[16], malondialdehyde, MDA[19] and nitric oxide, NO[20] were determined in serum according to previously described methods.

Statistical Analysis
Data were expressed as Mean ± SD and analyzed with, ANOVA, correlation and Tukey HSD post hoc using Microsoft Excel (2013). Values were considered significant at $p<0.05$. Correlation coefficients between 0.68 and 1.0 were considered highly correlated (+++), coefficients between 0.36 and 0.27 were considered moderately correlated (++) and coefficients <0.35 were considered weakly correlated (+). Correlation coefficients <0 possess zero (-) correlation[21].

Results and Discussion
Malarial infection is associated with the assault of red blood cells (RBCs) and the generation of large amounts of reactive oxygen species (ROS), therefore, producing oxidative stress[2]. Increased lysis of RBCs is associated with reduced packed cell volume (PCV) and haemoglobin[22] leading to anaemia found in severe malaria. Antioxidant enzymes exist to counteract the adverse effects of ROS[23]. However, in malaria, this is not the case as there exists a redox imbalance between ROS generation and neutralization by enzyme antioxidant system[2]. Other hematological parameters including white blood cell count, which should be increased in conditions requiring its phagocytic action, are not affected by malaria[24]. This could be as a result of the presence of excess ROS alongside the failing antioxidant enzyme defense system. Prolonged infection causing severe malaria could result in life-threatening complications including, tissue damage[25]. The many complications and failing therapy associated with malaria pose a threat for its eradication. Our study, therefore, determines the acute oral toxicity and the *in vivo* antiplasmodial activity of phytochemicals of *P. amarus* and its specific activities on antioxidant enzymes and nitric oxide (NO) concentrations in malaria infected mice.

The *in vivo* antiplasmodial activity of *P. amarus* phytochemical extracts was evaluated in this study by assessing the antimalarial activity during entrenched infection in rodent model. Daily progression of parasitaemia in experimental mice treated with 100, 300 and 500 mg/kg of *P. amarus* phytochemicals are displayed in Figures 1. The figure show that at day 12 (*i.e.* 6 days after the 4-day treatment with *P. amarus* phytochemicals or Lonart®DS), parasitaemia in experimental mice had declined significantly ($p<0.05$). Parasitaemia reduction was dose dependent and was highest at 500 mg/kg of the alkaloid dose.

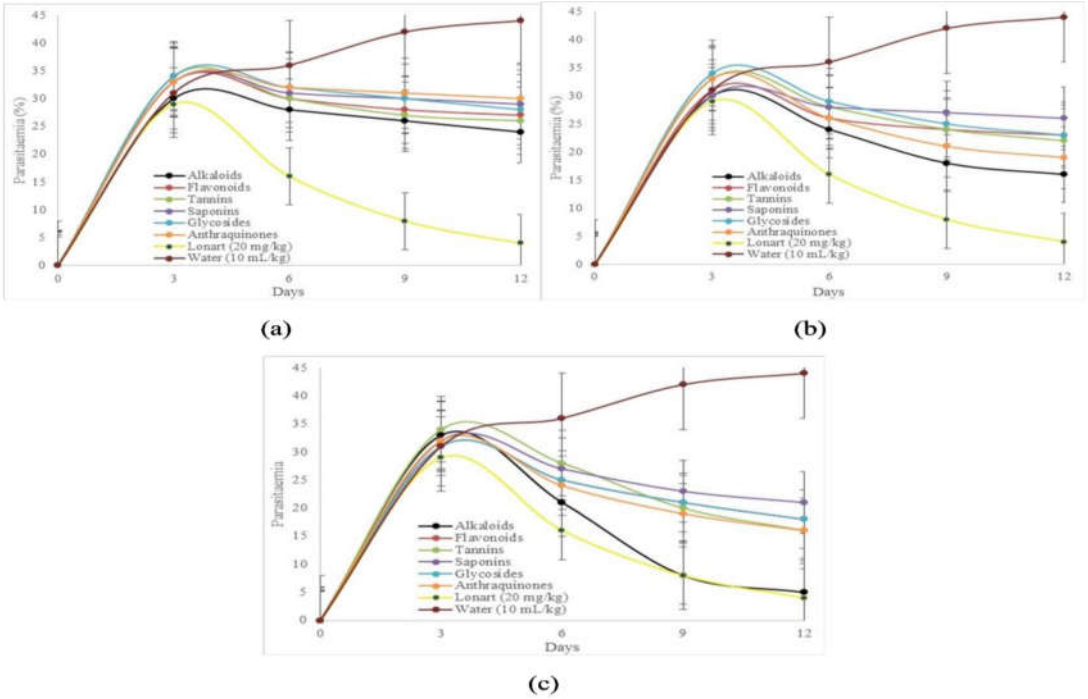

Figure 1: Changes in parasitaemia in mice treated with 100 mg/kg (a), 300 mg/kg (b) and 500 mg/kg (c) of phytochemicals of *P. amarus* assessed at day 3 (confirmation of parasitaemia and commencement of the 4-day treatment), day 6 (end of treatment), day 9 (third day of post treatment) and day 12 (sixth day of post treatment).

Parasite suppression calculated from parasitaemia values obtained at Day 12 are shown in Table 1. Phytochemical analysis showed that alkaloids are the most abundant phytochemical in leaves of *P. amarus*, while, the least present phytochemical is anthraquinones (Table 1). As shown in Table 1, all doses of phytochemicals of *P. amarus* showed a dose dependent increase in parasite suppression. However, suppression by the highest dose (500 mg/kg) of the alkaloid phytochemical was notable and showed no significant difference when compared with the activity of the standard drug in blood ($p>0.05$). The antiplasmodial activity of *Phyllanthus amarus*, the plant in this study, has been reported several times by previous studies[8,14,26]. Our study further confirms this activity and suggests that the antimalarial activity of the extracts resides essentially in the activity of the alkaloids.

Table 1: Phytochemical yield and parasite suppression induced by the phytochemicals of *Phyllanthus amarus* after six days of post treatment period

Phytochemical	Yield (%)	Doses (mg/kg)	Parasite suppression (%)
Lonart® DS	-	20	91±5.1[a]
Negative control	-	10	46±5.4[b]
Alkaloids	4.1	100	46±5.4[b]
		300	63±4.8[c]
		500	88±5.2[a]
Flavonoids	1.4	100	38±3.1[b]
		300	47±3.4[b]
		500	58±2.9[c]
Tannins	2.5	100	41±3.4[b]
		300	52±3.1[c]
		500	63±2.9[c]
Saponins	2.2	100	33±3.2[b]
		300	42±4.1[b]
		500	53±3.6[c]
Glycosides	1.6	100	36±3.3[b]
		300	47±3.6[b]
		500	51±3.4[c]
Anthraquinones	1.1	100	33±2.8[b]
		300	54±3.2[c]
		500	64±3.0[c]

Values are expressed as Mean ± SD for n = 5/group. Values with different superscript in columns differ significantly at $p<0.05$.

Acute oral toxicity assay showed no toxic attributes of the phytochemical of *P. amarus* (Table 2), even at the limit dose (5,000 mg/kg). All phytochemicals of *P. amarus* are, therefore, non-toxic with LD_{50} >5000 mg/kg. This is also consistent with other reports from other researchers who documented the non toxicity of extracts of *P. amarus* in both *in vivo* and *in vitro* systems[8,26].

Table 2: Oral acute toxicity of *Phyllanthus amarus phytochemicals*

Phytochemicals	Doses (mg/kg)						
	0	10	100	1000	2000	3000	5000
Alkaloid							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None
Flavonoid							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None
Tannins							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None
Saponins							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None
Glycosides							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None
Anthraquinone							
• Dead/Survivor	0/5	0/5	0/5	0/5	0/5	0/5	0/5
• Toxicity signs	None	None	None	None	None	None	None

Plasmodium infection was marked by significant (p<0.05) reduction in packed cell volume (PCV), red blood cells (RBC), haemoglobin (Hb) and significantly increased (p<0.05) monocytes (Mono) and neutrophils (Neu) in experimental mice. There was no significant (p>0.05) change in white blood cells (WBC) when comparing negative control with positive control group (Table 1).

Haematological parameters from our study showed that malarial infection presented anaemia in experimental mice. Our study reports significantly reduced PCV, RBC, and Hb with increased monocytes. These observations align with the report of other researchers. Reduced PCV, Hb, RBC[22] and increased monocytes[27] have been reported in humans infected with *Plasmodium falciparum*. Decreased RBC is the most common adverse hematology effect associated with malaria. Reduced RBCs has been related to the observation that healthy RBCs in malaria patients tend to have a shorter life span in comparison with healthy RBCs in healthy individuals.

Their haemolysis seems to account for more than 90% of erythrocyte loss during acute malaria[28]. Oxidative stress plays an important role in the development of malarial anaemia[29] thus, making anaemia a reliable predictor of the severity of disease. Rapid hemolysis of RBCs (infected and non-infected) is the major cause of decreased haemoglobin[30] which would be accompanied by a complementary reduction in PCV. Administration of the phytochemicals of *P. amarus* alleviated the effects of malaria on these parameters (RBC, PCV and Hb). This study can be related to the study of Nwakpa et al[31] who reported an increased haematological parameters (RBCs, Hb, PCV) in mice infected with *Samonella typhi* and treated with extract of *P. amarus*. Kolawole et al[32] also reported an increase in RBCs and WBCs in healthy mice treated with the aqueous leaf extract. Parameters that showed no significant alteration (WBCs and neutrophils) following malaria infection in this study, were negatively affected by treatment with *P. amarus* phytochemicals. Significantly altered WBC and neutrophils were reported in this study. This may be as a result of the adverse effect of malaria and alongside treatment with *P. amarus*.

Table 3: Effect of phytochemicals of *Phyllanthus amarus* on hematological parameters

Treatments	Doses (mg/kg)	Hematology					
		PCV (%)	WBC (x10^{12}/L)	RBC (x10^{12}/L)	HB(g/dL)	NEU (%)	MONO (%)
Control (NINT)	-	37.0±1.6^{a}	7.9±2.9^{a}	6.2±0.3^{a}	12.3±0.5^{a}	5.3±1.8^{a}	7.8±1.3^{a}
Standard drug	20	33.5±2.5^{a}	6.9±0.6^{a}	5.6±0.4^{b}	11.2±0.8^{a}	5.0±2.0^{a}	7.5±0.5^{a}
Negative control	-	23.0±1.9^{b}	6.3±1.5^{a}	3.8±0.3^{c}	7.7±0.6^{c}	6.8±2.2^{b}	15.0±2.0^{b}
Flavonoids	100	29.3±1.5^{b}	7.3±1.9^{a}	4.9±0.3^{d}	9.8±0.5^{b}	11.5±2.5*	10.8±2.3^{c}
	300	34.5±1.1^{a}	10.3±1.7*	5.8±0.2^{b}	9.5±0.8^{b}	9.3±2.2*	19.8±1.2$^{\#}$
	500	35.5±1.5^{a}	6.9±0.6^{a}	5.9±0.7^{b}	11.8±0.5^{a}	9.5±2.6*	9.0±1.4^{c}
Saponins	100	31.5±3.2^{a}	8.1±0.9^{a}	5.3±0.5^{b}	10.5±1.1^{b}	8.3±2.3*	9.8±1.1^{c}
	300	32.3±1.8^{a}	8.2±2.3^{a}	5.4±0.3^{b}	10.7±0.6^{b}	9.0±2.4*	6.0±2.4^{a}
	500	33.8±2.6^{a}	6.0±0.8^{a}	5.6±0.4^{b}	11.3±0.9^{a}	12.5±1.1*	3.3±2.2*
Glycosides	100	28.8±2.2^{b}	6.7±0.7^{a}	4.8±0.4^{d}	9.6±0.7^{b}	6.3±2.1^{a}	4.7±2.4*
	300	32.5±2.9^{a}	6.6±1.7^{a}	5.4±0.5^{b}	9.1±0.6^{b}	5.8±2.6^{a}	7.5±0.9^{a}
	500	31.8±3.9^{a}	7.8±1.8^{a}	5.3±0.7^{b}	9.9±0.0^{b}	8.3±1.1*	8.0±1.0^{a}
Alkaloids	100	31.0±4.5^{a}	6.0±1.2^{a}	5.1±0.8^{b}	10.3±1.5^{b}	11.7±2.4*	7.4±2.8^{a}
	300	31.0±3.6^{a}	3.9±0.5$^{\#}$	5.1±0.6^{b}	10.3±1.2^{b}	8.2±2.2*	2.7±0.3*
	500	34.3±4.3^{a}	5.7±0.9$^{\#}$	5.7±0.7^{b}	11.4±1.4^{a}	14.8±2.9*	3.6±2.6*
Anthraquinones	100	30.5±3.2^{a}	7.4±1.7^{a}	5.0±0.4^{d}	10.2±1.0^{b}	8.3±4.1*	8.8±0.4^{a}
	300	34.3±0.4^{a}	7.0±0.7^{a}	5.7±0.1^{b}	11.4±0.2^{a}	7.4±2.8*	11.0±2.7^{c}
	500	30.8±1.8^{a}	4.4±0.9$^{\#}$	5.3±0.5^{b}	10.6±0.9^{b}	10.5±2.2*	15.0±2.2^{b}
Tannins	100	38.3±4.0^{a}	5.3±0.7$^{\#}$	6.1±0.4^{a}	12.2±0.9^{a}	10.8±2.9*	8.5±2.9^{a}
	300	34.2±1.3^{a}	5.3±1.7$^{\#}$	5.4±0.8^{b}	10.8±1.6^{b}	9.8±2.1*	14.4±1.1^{b}
	500	32.8±3.3^{a}	7.7±1.2^{a}	4.8±0.7^{d}	9.7±1.4^{b}	8.3±2.9*	7.8±1.3^{a}

Values are expressed as Mean ± SD for n = 5/group. Values bearing the same letter superscript are not significantly different (p>0.05). $^{\#}$=significant decline compared with negative control. *=significant increase compared with positive control. NINT = Not infected, not treated.

The action of oxidative stress during malarial infection is unclear, some researchers demonstrated a protective role while others have confirmed a relation with malarial pathology[33]. To effectively analyse its pathology, we measured catalase (CAT), reduced glutathione (GSH), glutathione- S- transferase (GST), glutathione peroxidase (GPx), malondialdehyde (MDA), myeloperoxidase (MPO) and superoxide dismutase (SOD) levels in serum of infected mice treated with phytochemicals of *P. amarus* and its correlation to parasite suppression by same phytochemicals. Lipid peroxidation indicated by the increased levels of MDA, a biomarker of oxidative stress, is greatly elevated in untreated *P. berghei* infected mice[32]. Elevated MDA levels in *Plasmodium* infection has been confirmed by other researchers[34,35]. The increased amount of MDA generated during malaria infection may be caused by activation of the immune system[33]. However, our study indicates that there is a negative correlation between parasite suppression and MDA levels. It is known that a strong

antioxidant system will prevent excessive formation of MDA. Hence, all phytochemicals extracts of *P. amarus,* especially the flavonoid phytochemical, possess significant antioxidant activity.

Catalase protects body cells from oxidative stress by converting hydrogen peroxide to water and oxygen[36]. From our results, we could conclude that there is a general strong positive correlation between parasite suppression and catalase increase in blood of *P. berghei* infected mice treated with *P. amarus*. Increased catalase activity was very notable in flavonoid treated mice. Since parasite suppression is inversely related to parasitaemia, we can conclude that there exists a negative correlation between parasitaemia and catalase activity. This report agrees with the study of Sakyi et al[34] who reported decrease catalase concentrations in children with severe *P. falciparum* infection. Bilgin et al[36] also reported reduced catalase activity in *P. vivax* patients and relates this activity to low rates of H_2O_2 diffusion in *P. vivax* infected patients. Another study suggests that catalase activity might be affected by the endocytosis of the erythrocyte cytoplasm by *P. vivax*, therefore, accordingly, due to haemoglobin consumption, parasites might capture other proteins as antioxidant enzymes[33].

Decreased concentrations of nitric oxide (NO) has been reported in cases of severe malaria. Cramer et al[37] reported protection against severe malarial disease (oxidative stress) in patients having high blood levels of NO, it may contribute to pathophysiology of cerebral malaria and severe anaemia. This is because nitric oxide synthase activity is inhibited in cerebral malaria, a severe manifestation of malaria[38]. Inhibition is as a result of haemozoin interactions with L-arginine, a precursor of NO, reducing its availability for nitric oxide synthase, hence reducing nitric oxide production and bioavailability[39]. A study showed that administration of NO as an adjunctive for malaria therapy protected mice from cerebral malaria by improvement of brain microcirculatory hemodynamics and decreased vascular pathology[40]. Therefore, agents that induce increased production or activity of NO could be considered as antimalarials. Our study shows that the alkaloid phytochemical of *P. amarus* impressively induced increased concentrations of NO further confirming its antiplasmodial activity.

Table 4: Changes in blood antioxidants induced by the treatment of malarial infected mice with phytochemicals of *Phyllanthus amarus*

Treatment	Doses (mg/kg)	Antioxidants							
		CAT (Unit/mg protein)	GPx (Unit/mg protein)	GST (Unit/mg protein)	SOD (Unit/mg protein)	MPO (Unit/mg protein)	GSH (nmol/mg wet tissue)	MDA (nmol/mg wet tissue)	NO (μM)
Control (NINT)	-	102.4 ± 10.6^{a}	112.6 ± 12.3^{a}	82.3 ± 9.3^{a}	106.3 ± 12.7^{a}	15.3 ± 3.1^{a}	66.3 ± 5.8^{a}	2.2 ± 1.4^{a}	127.3 ± 13.4^{a}
Negative control	-	68.5 ± 7.5^{b}	63.3 ± 8.4^{b}	43.4 ± 6.3^{c}	51.2 ± 6.8^{b}	46.4 ± 5.2^{b}	48.4 ± 6.7^{b}	16.3 ± 2.1^{c}	63.4 ± 9.6^{c}
Standard drug	20	88.1 ± 11.4^{c}	94.3 ± 7.2^{c}	69.2 ± 8.5^{b}	91.3 ± 8.4^{c}	34.3 ± 3.5^{c}	52.3 ± 6.3^{b}	5.2 ± 1.2^{b}	86.7 ± 7.2^{b}
Flavonoids	100	$72.3\pm6.8^{b(+++)}$	$86.4\pm8.3^{c(+++)}$	$46.3\pm6.4^{c(+++)}$	$89.4\pm8.4^{c(+++)}$	$38.3\pm4.6^{c(-)}$	$50.6\pm6.3^{b(-)}$	$4.6\pm1.2^{b(-)}$	$86.3\pm6.7^{b(+++)}$
	300	93.5 ± 7.2^{a}	83.3 ± 7.2^{c}	63.2 ± 5.6^{b}	96.3 ± 8.9^{c}	32.4 ± 3.8^{c}	47.2 ± 5.7^{b}	3.8 ± 1.1^{a}	92.5 ± 6.8^{b}
	500	96.3 ± 8.3^{a}	92.4 ± 6.3^{c}	72.8 ± 6.4^{b}	98.6 ± 9.2^{c}	35.6 ± 4.1^{c}	48.2 ± 5.3^{b}	3.2 ± 1.3^{a}	103.7 ± 7.2^{b}
Saponins	100	$69.2\pm7.4^{b(++)}$	$81.3\pm7.6^{c(+++)}$	$51.3\pm5.3^{c(-)}$	$87.3\pm7.3^{c(+++)}$	$40.3\pm5.2^{b(-)}$	$46.3\pm3.9^{b(++)}$	$4.1\pm1.4^{b(+++)}$	$91.7\pm6.6^{b(++)}$
	300	83.4 ± 7.8^{c}	87.4 ± 8.2^{c}	56.4 ± 4.8^{c}	94.6 ± 6.9^{c}	33.3 ± 4.1^{c}	45.4 ± 4.8^{b}	4.4 ± 1.2^{b}	87.7 ± 7.3^{b}
	500	80.7 ± 6.6^{c}	90.3 ± 7.9^{c}	49.4 ± 5.1^{c}	97.2 ± 7.2^{c}	36.2 ± 3.3^{c}	48.5 ± 6.1^{b}	4.8 ± 1.6^{b}	98.3 ± 7.5^{b}
Glycosides	100	$75.6\pm10.2^{c(++)}$	$83.2\pm6.8^{c(-)}$	$48.2\pm4.5^{c(++)}$	$90.3\pm9.2^{c(-)}$	$41.6\pm2.9^{b(-)}$	$49.3\pm3.9^{b(+++)}$	$4.8\pm2.1^{b(-)}$	$82.3\pm6.8^{b(+)}$
	300	94.2 ± 9.6^{a}	85.3 ± 7.1^{c}	59.3 ± 5.1^{b}	96.4 ± 8.6^{c}	33.4 ± 3.2^{c}	54.2 ± 4.1^{b}	3.6 ± 1.8^{a}	88.5 ± 5.9^{b}
	500	88.3 ± 7.4^{c}	80.2 ± 6.3^{c}	53.5 ± 4.3^{c}	83.5 ± 7.3^{c}	38.6 ± 3.7^{c}	58.3 ± 4.3^{a}	4.4 ± 1.6^{b}	85.4 ± 6.1^{b}
Alkaloids	100	$63.4\pm7.2^{b(+)}$	$81.2\pm7.2^{c(+)}$	$40.3\pm2.8^{c(+++)}$	$76.3\pm7.4^{c(++)}$	$41.3\pm4.2^{b(-)}$	$48.3\pm4.1^{b(-)}$	$4.9\pm2.1^{b(+++)}$	$110.6\pm11.3^{a(+++)}$
	300	72.3 ± 6.4^{b}	72.6 ± 6.9^{b}	46.4 ± 2.5^{c}	84.5 ± 6.8^{c}	32.4 ± 3.1^{c}	42.4 ± 4.3^{b}	5.2 ± 2.3^{b}	120.3 ± 10.6^{a}
	500	66.7 ± 7.2^{b}	86.5 ± 7.8^{c}	45.3 ± 2.7^{c}	80.3 ± 7.1^{c}	38.3 ± 3.8^{c}	$39.3\pm3.8^{\#}$	5.4 ± 2.2^{b}	125.6 ± 11.7^{a}
Anthraquinones	100	$71.3\pm6.7^{b(++)}$	$84.4\pm5.8^{c(-)}$	$46.2\pm4.5^{c(+++)}$	$82.9\pm6.8^{c(+++)}$	$39.2\pm3.8^{b(-)}$	$50.1\pm3.8^{b(-)}$	$4.7\pm1.3^{b(-)}$	$87.8\pm9.2^{b(++)}$
	300	87.4 ± 6.8^{c}	78.5 ± 6.1^{b}	51.3 ± 4.1^{c}	88.2 ± 7.1^{c}	33.8 ± 2.6^{c}	46.3 ± 4.1^{b}	4.3 ± 1.2^{b}	100.1 ± 8.4^{b}
	500	82.3 ± 7.1^{c}	82.3 ± 5.9^{c}	55.4 ± 4.8^{c}	90.3 ± 7.3^{c}	37.2 ± 3.2^{c}	51.2 ± 4.3^{b}	4.1 ± 1.6^{b}	92.4 ± 7.8^{b}
Tannins	100	$71.3\pm5.8^{b(+++)}$	$82.4\pm6.1^{c(-)}$	$43.2\pm4.4^{c(+++)}$	$84.3\pm6.5^{c(-)}$	$40.3\pm2.8^{b(+)}$	$52.2\pm5.1^{b(++)}$	$4.8\pm2.1^{b(-)}$	$86.7\pm8.2^{b(+++)}$
	300	76.8 ± 6.1^{c}	76.3 ± 5.8^{b}	50.3 ± 4.6^{c}	89.4 ± 7.1^{c}	36.4 ± 3.6^{c}	48.1 ± 4.8^{b}	4.1 ± 1.2^{b}	89.4 ± 6.8^{b}
	500	80.4 ± 6.8^{c}	80.4 ± 5.3^{c}	53.4 ± 4.1^{c}	80.3 ± 7.3^{c}	44.5 ± 3.3^{b}	59.2 ± 4.1^{a}	3.8 ± 1.0^{a}	96.8 ± 5.6^{b}

Values are expressed as Mean ± SD for n = 5/group. Values bearing the same letter superscript are not significantly different (p>0.05). #=significant decline compared with negative control. Correlation between parasite suppression and enzyme activity are indicated by (+++), (++), (+) and (-) superscripts. +++ indicates strong correlation, ++ indicates weak correlation, + indicates very weak correlation and – indicates zero (no) correlation. NINT = Not infected, not treated.

Prabhu et al[35] showed that there is a negative correlation between GPx levels and MDA levels in *P. vivax* and *P. falciparum* infected patients. This is consistent with our present study which also confirms reduced GPx levels and increased MDA levels in *P. falciparum* mice. Oluba[41] also reports reduced GPx and GSH levels in children infected with *P. falciparum*. Reduced GST levels in malarial infection has also been reported[42]. Reduced concentration of SOD in malaria infection has been reported[36,41] and these are consistent with results obtained from our study. Myeloperoxidase (MPO), a heme protein, is secreted by activated neutrophils in inflammatory disease as an immune response[43]. Therefore, there exist a correlation between increased neutrophils and MPO enzymes in severe *P. falciparum* infection[44]. This is consistent with our study that reveals increased MPO and neutrophil concentrations following *P. berghei* infection. Thee et al[43] suggests that MPO modulates the adaptive immune response during malaria infection, leading to an attenuated parasite clearance.

Therefore, neutrophil activation and subsequently, increased myeloperoxidase activity are implicated in the pathogenesis and severity of malarial infection. However, no phytochemical extract reduced neutrophil and MPO concentrations, suggesting that this may not be the mechanism of action.

Overall, malarial infection produced a significant decrease in antioxidant markers with the exception of MPO and MDA which were significantly increased, when indices in negative control groups were compared with positive control group. There was also significantly reduced nitric oxide concentrations in malarial infected mice. However, administration of phytochemicals of *P. amarus* enhanced enzyme production and/or activity. Overall, improvement in antioxidant index was achieved by the flavonoid phytochemical as opposed to alkaloids, which produced highest antiplasmodial activity. But, improvement in nitric oxide concentrations were greatest in alkaloid phytochemical extract treated mice.

Conclusion

Six phytochemicals (flavonoids, saponins, glycosides, alkaloids, anthraquinones and tannins) were identified, extracted and quantified. They were non-toxic and alkaloid was most abundant. The flavonoid extract demonstrated the highest antioxidant activity, while the alkaloid, the most reputable antimalarial action.

The study therefore, suggests that antioxidant activity may not be the major mechanism responsible for the demonstrated antimalarial activity of the alkaloid extract of *P. amarus*. However, the high amounts of nitric oxide (NO) produced by alkaloid treatment possibly suggests low concentrations of haemozoin which interacts with L-arginine, the precursor molecule of NO. Therefore, one probable mechanism may involve inhibition of haem polymerisation to haemozoin. Haem, therefore, accumulates and destroys the *Plasmodium* parasite. Antioxidants may be important in defending the hosts erythrocytes against the oxidative assault during malarial infection and hence, complement treatment to be more effective. Therefore, the outcome of alkaloid and flavonoid co-treatment on haem metabolism should be investigated.

Declaration of interest statement

The authors declare that there is no conflict of interests.

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Authors Contribution

TE, JOA and PEA, conducted the laboratory experiments as designed. COE analyzed and interpreted the data. She also prepared the draft manuscript which was approved by all authors for submission. IO conceived and designed the study. He also supervised the research at every stage and vetted the draft manuscript for approval and submission.

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