



Levels of Cerebral Malaria Indices, Free radical Scavengers and Preventive Antioxidants in *Plasmodiumberghei* Infected Mice Treated with Phytochemical Extracts of *Phyllanthus amarus* Schum and Thonn

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Abstract

Cerebral malaria (CM) is one of the deadliest form of severe malaria, caused by prolonged oxidative stress and characterized by neurological dysfunctions. Antioxidant treatment in conjunction with antimalarials could provide an alternate chemotherapeutic pattern to curb oxidative stress associated with CM. Therefore, this study profiled the phytochemicals of *Phyllanthus amarus* and evaluated their specific activities on decreasing CM indices in experimental mice. A hundred and five (105) adult mice were divided into 3 control groups and 18 test groups each containing 5 mice. Having confirmed parasitaemia in all groups, except positive control group, which was left uninfected, all test groups were treated with varying doses (100, 200 and 400 mg/kg) of phytochemical (alkaloid, flavonoid, saponin, tannin, anthraquinone and glycoside) extracts of *P. amarus*. Then, standard control group was treated with Lonart[®]DS, containing artemether and lumefantrine. Antiplasmodial activity, CM indices (parasite count, chemosuppression and glucose concentrations), preventive antioxidant levels (myoglobin and ferritin), free radical enzyme scavengers' (SOD, CAT, GPx, GSH, GST, MPO, MDA) activities and nitric oxide (NO) levels in blood and brain tissues were assessed using documented methods. Results from the study show that infection of experimental mice with *P. berghei* (NK 65) malarial parasite induced CM within nine days of inoculation. The CM was observed to be associated with brain parasites, anaemia, reduced brain NO and increased cerebral glucose and oxidative status. However, treatment of the induced infection with either the standard drug (Lonart[®]DS) or phytochemical extracts ameliorated the observed disturbances with the alkaloid extract having the most significant impact in averting CM and associated complications.

Keywords: Anaemia, severe malaria, flavonoids, alkaloids, *P. amarus*, haematological indices

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Introduction

The commonest, most important complications of *Plasmodium falciparum* infection in children are cerebral malaria (CM), severe anaemia, respiratory distress (acidosis) and hypoglycemia. Although, all complications may manifest at once or individually, severe malaria cannot be ruled out with a confirmation of the absence of one of the above complications, since severe malaria can manifest in other forms (WHO, 2012). Severe malaria is due to prolonged oxidative stress caused by parasites invasion of erythrocytes producing large amounts of reactive oxygen species (Becker et al., 2004). Parasitized erythrocytes cause tissue dysfunction by adherence to endothelial cells and circulating immune cells by interactions between specific ligands on parasitized erythrocytes, hosts adhesion receptor and intracellular adhesion molecules (Ockenhouse et al. 1991). This leads to inflammatory cytokine secretion, platelet aggregation and expression of adhesion molecule and tissue factor (Francischetti et al. 2008).

CM is one of the most lethal manifestation of the disease affecting mostly the younger population of under five years of age (Lesi et al. 2005). This occurs as a result of sequestration of parasitized erythrocytes causing increased permeability of the blood brain barrier (Francischetti et al. 2008). Manifestations of cerebral malaria leads to a progressive loss of cognitive abilities, seizures, coma and eventually, death (Newton et al. 2000). Survivors of cerebral malaria may display neurologic deficits even after 2 years of post infection and treatments (Oluwayemi et al. 2013). Olumese et al. (1999) reported that children presenting acute encephalopathy in malaria-endemic areas should be considered for careful clinical observations.

The use of antimalarials alone cannot curb the disastrous outcomes associated with cerebral malaria (Ackerman et al., 2009). Despite improved survival with artesunate treatment (Dondorp et al. 2005), mortality remains high, and therefore, adjunctive therapies are still urgently needed. Although, most antimalarials act as pro-oxidants, increasing the oxidative stress alongside the *Plasmodium*, the use of antioxidants in reducing the risk and severity of cerebral malaria has been documented.

Phyllanthus amarus has been reported to possess significant antioxidant activity (Adeneye et al. 2006). Oshiegbu et al. (2022), have reported its ability in reducing malaria-induced oxidative stress in reproductive system of male mice. Karuna et al. (2011) and Onyesom et al. (2015) reported that aqueous extract of *P. amarus* reduced renal oxidative stress. Nevertheless, the impact of *P. amarus* on cerebral malaria and brain antioxidant status are not well known. Therefore, in this present study, we profiled the phytochemicals of *P. amarus* and evaluated their impact on cerebral malaria indices, preventive antioxidants, free radical scavengers and nitric oxide concentrations in malarial infected mice treated with graded doses of the phytochemical extracts of *P. amarus*.

Materials and Methods

Collection of Plant Materials and Preparation of Phytochemical Extracts

Leaves of *Phyllanthus amarus* were collected, washed and air dried at laboratory room temperature and thereafter crushed using a laboratory blender (Kenwood, Japan). The phytochemicals; alkaloid (Uzuegbu et al. 2020), tannin (Ukoha et al. 2011), flavonoid (Cai et al. 2010), saponin (Klujanabagavad and Wmk, 2009), anthraquinone (Bruneton, 1995) and glycoside (Sharma et al. 2014) were identified and extracts were prepared by standard methods (already described).

Experimental Animals

Eight week old adult Swiss albino mice (BALB/c albino strain; 22-28 g b.wt) were obtained from Laboratory Animal Centre, LAC, Faculty of Basic Medical Sciences, FBMS, Delta State University, DELSU, Abraka, Nigeria.



Parasites and Inoculation of Experimental Animals

Plasmodium berghei (Strain NK65) parasites already inoculated in donor mice were procured from the Department of Parasitology, Nigerian Institute of Medical Research, NIMR, Yaba, Lagos State, Nigeria.

A hundred and five (105) mice were selected for this study. Mice were segregated into twenty-one groups, three control groups i.e. positive, negative and standard controls, and eighteen test groups (5 mice per group). Mice in positive control group were neither infected nor treated, negative control group were infected and not treated and standard control group was infected and treated with 20 mg/kg of standard drug, artemether and lumefantrine (Lonart®DS). Test groups were further segregated into phytochemical groups based on dosage (100, 200 and 400 mg/kg for each phytochemical). Dosage was selected based on previous studies (Oshiegbu et al. 2022).

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.

Antimalarial activity

After inoculation, *P. amarus* phytochemical extracts and standard drug were administered once daily for a period of four days via intragastric cannula. Parasitaemia was assessed at Day 0, 3, 6, 9 and 12 using blood collected from the cut tail tip of infected mice. Blood collected were prepared by making blood smears, stained with Giemsa and viewed under the microscope at x40 magnification (Oshiegbu et al. 2022). Parasitaemia and chemosuppression were calculated using the following formula:

$$\% \text{ parasitaemia} = (\text{Number of parasitized red blood cell} / \text{Total number of red blood cell}) \times 100$$

$$\% \text{ chemosuppression} = [(\text{Mean parasitaemia of negative control} - \text{Mean parasitaemia of treated group}) / \text{Mean parasitaemia of negative control}] \times 100$$

Tissue (brain) collection and processing

On Day 12 (i.e. 6th day of post treatment) the mice were fasted overnight and euthanized. Thereafter, the brain was collected and processed as required for analysis. One gram (1.0g) of the fresh tissue was blended and homogenized in 49ml of normal saline. The homogenate was then centrifuged (80D Serico, China) at 12000xg for 15min at room temperature (28-30°C) to obtain the supernatant. The supernatant was transferred into another plain sterile bottle and kept frozen until required for antioxidant assay.

To obtain parasite count for brain tissues, homogenate was used to make a smear on clean microscopic slides and allowed to dry. Dried smears were then stained with Giemsa stain and read with microscope. Parasites were counted and chemosuppression was then, calculated using aforementioned formula.

Glucose estimation

Glucose oxidase method was used to measure plasma and tissue (brain) glucose using reagent kit of Randox in test and control mice.

Haematological indices (PCV and Hb) estimation

Haematological indices were assessed using documented methods (Nwakpa et al., 2014). About 5ml of blood was collected from euthanatized mice by cardiac puncture with a sterile syringe and needle into EDTA treated screw-cap sample bottles. PCV and Hb were then, estimated using an hematological analyzer.

Ferritin and Myoglobin Estimation



Plasma myoglobin and ferritin concentrations were estimated by ELIZA technique in test and control mice using *in vitro* ELISA kit as designed for the accurate quantitative measurement of myoglobin and ferritin in serum.

Brain Antioxidant Activity Assays

Activities of antioxidants; catalase, CAT (Hadwan et al. 2016), glutathione peroxidase, GPx (Moron et al. 1979), glutathione S-transferase, GST (Moron et al. 1979), superoxide dismutase, SOD (Marzec-Wroblewska et al. 2011), Myeloperoxidase, MPO (Russell et al., 2017) and then, concentrations of reduced glutathione, GSH (Moron et al. 1979), and nitric oxide, NO (Bryan and Grisham, 2007) were determined using standard methods (previously documented).

Statistical Analysis

Data were expressed as Mean \pm SEM and analyzed with one way ANOVA and Turkey HSD post hoc using Microsoft Excel (2007). Values were considered significant at $p < 0.05$.

Results

Cerebral malaria indices are highlighted in Table 1. Judging from these results, malaria infection significantly increased glucose concentrations in brain tissues when compared with positive control. However, mice groups treated with 200 and 400 mg/kg alkaloid, glycoside and saponin phytochemical extracts significantly reduced brain glucose levels and showed significantly similar values ($p > 0.05$) to positive control.

Also, treatment with the phytochemical extracts of *P. amarus* significantly reduced parasite count. These were achieved by all doses of phytochemical extracts of *P. amarus*, but, greatest reduction was, nonetheless, achieved by the highest dose (400 mg/kg) of the alkaloid phytochemical extract. Likewise, significant chemosuppression were only achieved in mice treated with 400 mg/kg of the alkaloid phytochemical extracts of *P. amarus* and these results compared well to standard drug control group.

Table 1: Cerebral malaria indices in *Plasmodium berghei* infected mice treated with phytochemical extracts of *Phyllanthus amarus*

Groups/Treatment	Dose (mg/kg)	Parasite Count (%)	Chemosuppression (%)	Glucose(mg/dL)
Positive Control (NINT)	-	0 \pm 0	-	92 \pm 6 ^a
Negative Control (INT)	-	25.0 \pm 4.0 ^a	0 \pm 0	110 \pm 8 ^b
Standard drug (Lonart [®] DS)	20	2.8 \pm 0.4 ^b	89 \pm 4 ^a	104 \pm 5 ^b
Alkaloids	100	13.0 \pm 1.6 ^c	48 \pm 3 ^b	102 \pm 6 ^b
	200	9.8 \pm 1.7 ^d	61 \pm 4 ^c	96 \pm 5 ^a
	400	6.3 \pm 1.1 ^d	75 \pm 5 ^a	90 \pm 7 ^a
Flavonoids	100	17.5 \pm 2.3 ^c	30 \pm 3 ^d	118 \pm 10 ^b
	200	14.8 \pm 2.8 ^c	41 \pm 3 ^b	112 \pm 8 ^b
	400	12.0 \pm 1.6 ^c	52 \pm 4 ^c	116 \pm 7 ^b
Tannins	100	18.0 \pm 1.4 ^c	28 \pm 3 ^d	123 \pm 11 ^b
	200	14.5 \pm 2.8 ^c	42 \pm 3 ^b	111 \pm 8 ^b
	400	11.5 \pm 1.4 ^c	54 \pm 4 ^c	115 \pm 9 ^b
Saponins	100	17.8 \pm 2.3 ^c	29 \pm 4 ^d	101 \pm 6 ^b
	200	15.3 \pm 2.1 ^c	39 \pm 4 ^b	98 \pm 5 ^a
	400	13.8 \pm 1.7 ^c	45 \pm 4 ^b	96 \pm 4 ^a
Glycosides	100	17.5 \pm 2.6 ^c	30 \pm 3 ^d	110 \pm 6 ^b



	200	16.3±2.5 ^c	35±4 ^d	94±7 ^a
	400	12.5±1.8 ^c	51±4 ^c	98±5 ^a
Anthraquinones	100	18.3±3.1 ^c	27±3 ^d	126±12 ^b
	200	17.4±2.6 ^c	31±3 ^d	121±11 ^b
	400	16.0±1.9 ^c	36±4 ^d	117±13 ^b

Data are expressed as Mean±SEM for n=5 mice per group. Values were taken on Day 12 (i.e. 6th day of post treatment). NINT= Not infected, not treated. INT= Infected, not treated. Values with different superscript in columns differ significantly at P≤0.05.

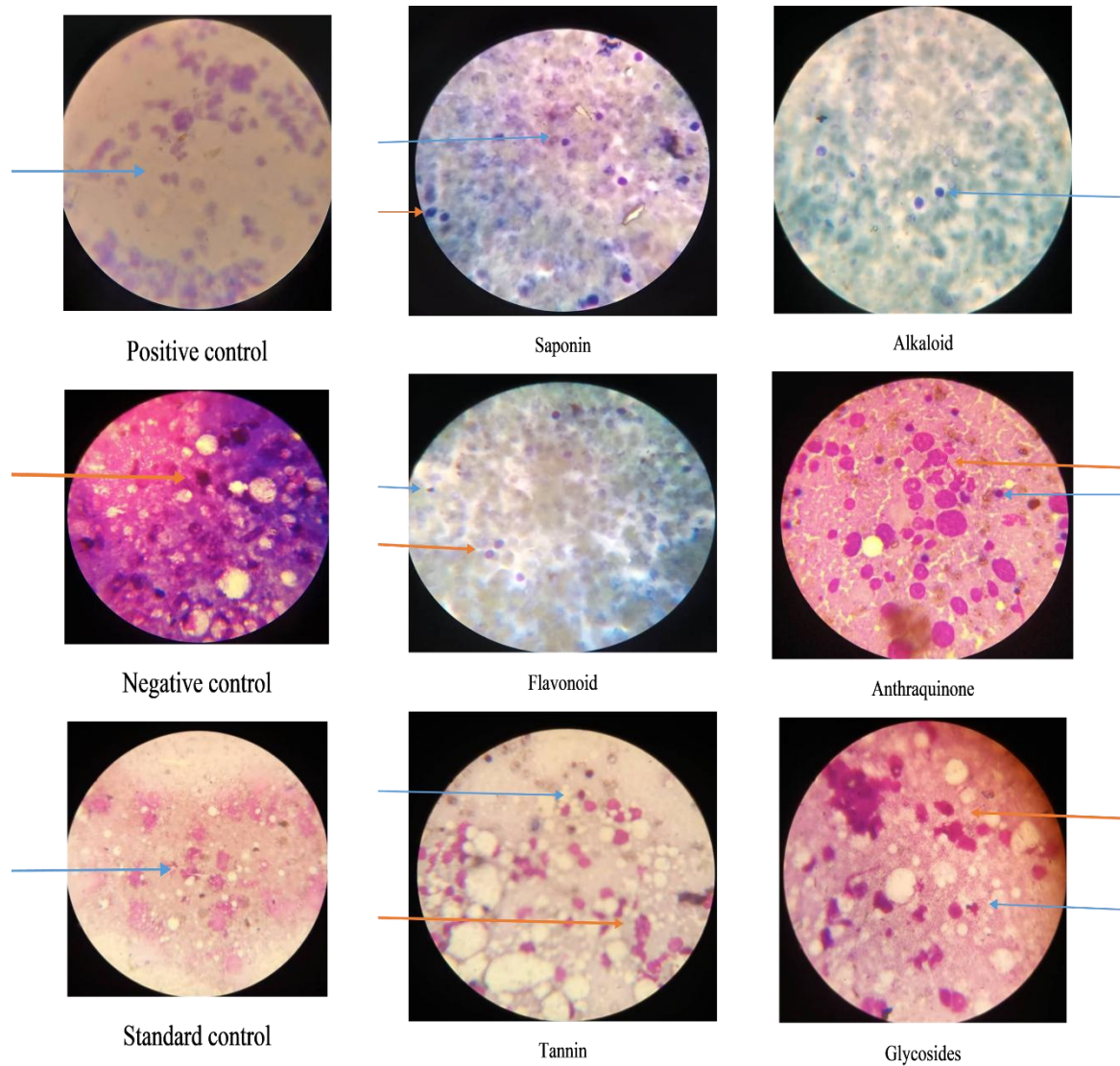


Figure 1: Brain films showing parasitized (red arrows) and normal (blue arrows) cells of uninfected (positive control), Infected without treatment (negative control) and Infected, but treated with standard drug (20 mg/kg Lonart®DS) or 400 mg/kg of each phytochemical (saponin, tannin, alkaloid, flavonoid, glycoside and anthraquinone) extract

The films confirm CM with varying proportion of parasite count, which showed that the standard drug and phytochemical extracts, particularly, the alkaloid extract, significantly suppressed CM.

Malarial indices, parasitaemia and chemosuppression, presented in Table 2, showed that all phytochemical extracts of *P. amarus* possess significant antimalarial activity. Activity of extracts



were dose dependent and greatest activity was attained by the mice treated with 400 mg/kg of alkaloids of *P. amarus*, which compared well with the standard drug control.

Malaria infection also produced significantly reduced blood glucose concentrations (Table 2). Post treatment with phytochemicals of *P. amarus* produced significant dose dependent increase in blood glucose levels. The activities of the alkaloid, tannin and saponin phytochemicals produced similar glucose concentrations with the positive and standard control groups at all dosage. Other phytochemical extracts, however, presented significantly higher glucose concentrations in comparison with the positive and standard controls.

Haematological indices, PCV and Hb, were significantly reduced in negative control mice as compared with positive control. With the exception of mice treated with 100 mg/kg of flavonoid and glycoside phytochemical extracts, all doses of the phytochemical extracts of *P. amarus* ameliorated these effects and presented similar values with the positive and standard control groups. Similar observations were also recorded for Hb index. Groups treated with 100 and 200 mg/kg of flavonoid and glycoside phytochemicals and 400 mg/kg of tannin phytochemical extracts produced statistically similar indices with negative control groups.

Preventive antioxidants, ferritin and myoglobin, were significantly increased in malaria infection. Generally, all dosage of the phytochemical extracts of *P. amarus* assuages the negative effect of malaria. Ferritin index statistically similar to the positive control group was only achieved by all dose groups of alkaloid and anthraquinone phytochemical extracts. However, no phytochemical extract was able to completely reverse the action of malaria infection on myoglobin concentrations, but the highest ameliorating activity was achieved by the alkaloid phytochemical.

Table 2: Malarial indices and preventive antioxidant (ferritin and myoglobin) status in blood of *P. berghei* infected mice treated with phytochemical extracts of *P. amarus*

Groups/Treatment	Dose (mg/kg)	Parasitaemia (%)	Chemosuppression (%)	Glucose (mg/dL)	PCV (%)	Hb (g/dl)	Ferritin (ng/mL)	Myoglobin (ng/mL)
Positive Control (NINT)	-	0±0	-	85±11 ^a	37±2 ^a	12±1.2 ^a	120±17 ^a	88±8 ^a
Negative Control (INT)	-	32.5±4.6 ^e	0±0	59±4 ^b	23±3 ^b	8±1.6 ^b	310±7 ^b	521±17 ^b
Standard drug (Lonart [®] DS)	20	2.9±0.7 ^a	91±6 ^a	71±7 ^a	34±4 ^a	11±1.8 ^a	183±11 ^c	256±12 ^c
Alkaloids	100	18.2±1.2 ^d	44±5 ^b	88±4 ^a	31±5 ^a	10±1.5 ^a	121±16 ^a	203±8 ^d
	200	12.7±0.8 ^c	61±6 ^c	86±5 ^a	32±4 ^a	11±1.2 ^a	110±7 ^a	184±10 ^e
	400	4.2±0.3 ^b	87±7 ^a	93±8 ^a	34±5 ^a	12±1.6 ^a	107±6 ^a	172±10 ^e
Flavonoids	100	20.5±2.3 ^d	37±4 ^b	103±11 ^c	29±2 ^b	8±0.5 ^b	274±18 ^e	296±13 ^f
	200	16.9±1.0 ^d	48±6 ^b	100±15 ^c	33±2 ^a	9±2.0 ^b	198±14 ^c	249±16 ^c
	400	14.6±1.1 ^c	55±5 ^c	102±8 ^c	35±3 ^a	10±1.3 ^a	157±23 ^d	212±13 ^d
Tannins	100	20.2±1.5 ^d	38±4 ^b	87±4 ^a	38±4 ^a	12±0.9 ^a	194±4 ^c	289±13 ^f
	200	16.6±1.2 ^d	49±3 ^b	96±7 ^a	34±2 ^a	11±2.1 ^a	163±13 ^d	258±11 ^c
	400	12.7±0.9 ^c	61±5 ^c	97±10 ^a	33±4 ^a	9±1.4 ^b	164±11 ^d	251±13 ^c
Saponins	100	22.8±2.2 ^d	30±6 ^d	88±4 ^a	32±3 ^a	10±1.1 ^a	198±5 ^c	211±13 ^d
	200	20.2±1.2 ^d	38±4 ^b	73±7 ^a	32±2 ^a	11±0.5 ^a	191±8 ^c	236±14 ^c
	400	15.9±1.6 ^d	51±5 ^c	88±6 ^a	34±5 ^a	11±0.9 ^a	163±17 ^d	256±13 ^c



Glycosides	100	21.8±2.7 ^d	33±4 ^d	111±15 ^c	29±3 ^b	9±0.7 ^b	309±14 ^b	276±14 ^f
	200	18.2±1.8 ^d	44±5 ^b	109±16 ^c	33±4 ^a	9±1.6 ^b	293±9 ^e	244±13 ^c
	400	14.6±1.5 ^d	55±3 ^c	93±9 ^a	32±5 ^a	10±2.1 ^a	317±12 ^b	258±12 ^c
Anthraquinones	100	23.4±1.2 ^d	28±2 ^d	101±9 ^c	31±3 ^a	10±1.1 ^a	125±13 ^a	277±13 ^f
	200	19.8±1.6 ^d	39±3 ^b	100±10 ^c	34±2 ^a	11±1.0 ^a	128±14 ^a	243±11 ^c
	400	16.9±1.8 ^d	48±4 ^b	106±8 ^c	31±2 ^a	10±0.9 ^a	125±11 ^a	257±10 ^c

Data are expressed as Mean±SEM for n=5 mice per group. Values were taken on Day 12 (i.e. 6th day of post treatment). NINT= Not infected, not treated. INT= Infected, not treated. Values with different superscript in columns differ significantly at P≤0.05.

Antioxidant enzymes' activities induced by the treatments of the malarial infection with the phytochemical extracts of *P. amarus* are summarized in Table 3. *P. berghei* malarial parasite infection in experimental mice significantly reduced SOD, GPx, GST, CAT, GSH and NO and increased MPO concentrations. However, treatment of the malarial induced infection with the graded doses of *P. amarus* phytochemical extracts ameliorated the disturbances in a dose-dependent manner.

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Table 3: Activities of free radical scavengers and nitric oxide levels in brain tissue of *P. berghei*-infected mice treated with phytochemical extracts of *P. amarus*

Groups/Treatment	Dose (mg/kg)	SOD (Unit/mg/Protein)	GPx (Unit/mg/Protein)	GST (Unit/mg/Protein)	CAT (Unit/mg/Protein)	MPO (Unit/mg/Protein)	GSH (mol/mg)	NO (µM)
Positive Control (NINT)	-	58±4 ^a	40±6 ^a	47±3 ^a	65±9 ^a	7±2 ^a	48±4 ^a	65±6 ^a
Negative Control (INT)	-	25±3 ^b	37±5 ^c	27±9 ^b	35±16 ^b	31±5 ^b	31±7 ^b	35±2 ^b
Standard drug (Lonart®DS)	20	43±5 ^c	43±7 ^a	49±4 ^a	46±5 ^c	23±3 ^c	38±5 ^b	56±8 ^d
Alkaloids	100	33±8 ^b	44±7 ^a	42±4 ^a	40±6 ^c	21±7 ^c	41±3 ^a	73±5 ^c
	200	42±5 ^c	61±5 ^b	49±6 ^a	43±7 ^c	23±9 ^c	43±6 ^a	69±3 ^a
	400	40±4 ^c	56±6 ^b	47±5 ^a	56±9 ^a	22±6 ^c	47±8 ^a	78±4 ^c
Flavonoids	100	34±4 ^b	55±6 ^b	46±3 ^a	33±6 ^b	26±4 ^c	35±9 ^b	58±7 ^d
	200	39±2 ^c	46±4 ^a	54±5 ^a	42±6 ^c	31±6 ^b	47±8 ^a	53±5 ^d
	400	50±3 ^a	43±5 ^a	52±4 ^a	54±7 ^c	23±7 ^c	50±5 ^c	52±3 ^d
Tannins	100	33±3 ^b	56±7 ^b	46±9 ^a	38±5 ^b	19±4 ^c	45±3 ^a	58±3 ^d
	200	38±4 ^c	58±9 ^b	44±8 ^a	51±4 ^c	21±6 ^c	47±5 ^a	60±5 ^a
	400	49±6 ^c	61±6 ^b	45±7 ^a	69±9 ^a	20±5 ^c	41±7 ^a	61±11 ^a
Saponins	100	32±4 ^b	47±3 ^a	39±3 ^b	43±9 ^c	22±4 ^c	54±7 ^c	62±5 ^a
	200	35±5 ^b	62±6 ^b	48±7 ^a	46±7 ^c	25±6 ^c	53±8 ^c	42±7 ^d
	400	42±6 ^c	60±5 ^b	50±6 ^a	51±7 ^c	23±7 ^c	47±9 ^a	58±6 ^d
Glycosides	100	32±6 ^b	52±6 ^b	42±4 ^a	39±11 ^b	22±3 ^c	43±3 ^a	57±5 ^d
	200	38±5 ^c	54±7 ^b	38±6 ^b	43±7 ^c	18±2 ^c	52±7 ^c	53±4 ^d
	400	47±6 ^c	62±8 ^b	36±5 ^b	56±10 ^a	23±6 ^c	53±8 ^c	65±7 ^a
Anthraquinones	100	35±5 ^b	53±6 ^b	41±7 ^a	50±7 ^c	23±4 ^c	40±6 ^a	66±7 ^a
	200	46±7 ^c	64±7 ^b	42±6 ^a	58±11 ^a	21±6 ^c	38±4 ^b	64±5 ^a
	400	42±6 ^c	55±4 ^b	39±5 ^b	53±8 ^c	22±5 ^c	42±8 ^a	58±8 ^d



Data are expressed as Mean±SEM for n=5 mice per group. Values were taken on Day 12 (i.e. 6th day of post treatment). NINT= Not infected, not treated. INT= Infected, not treated. SOD= Superoxide dismutase, GPx= Glutathione peroxidase, GST= Glutathione S-transferase, CAT= Catalase, MPO=Myeloperoxidase, GSH= Reduced glutathione, NO= Nitric oxide. Values with different superscript in columns differ significantly at P≤0.05.

Discussions

The severity of malaria is generally dependent on the immune system of the infected individual and the endemicity of the region the infection was acquired. This could be confirmed by the population of children and adults manifesting severe forms of the infection, as lesser adult population infected with malaria will develop severe malaria due to partial immunity (Benedette, 2021). The most common manifestation of severe malaria is anaemia (Anumudu et al. 2004), followed by cerebral malaria (Mavondo et al. 2019). Other rare, but reported manifestations include glucose abnormalities (Gildas et al. 2017), rhabdomyolysis (Yong et al. 2012), myoglobinuria (Mishra et al. 2010), acute kidney injury (Bachmann et al. 2014), acute lung injury (Mavondo et al. 2019) and retinopathy (Oliveira et al. 2017). These manifestations are as a result of over production of reactive oxygen species by the *Plasmodium* parasite infection whose pathogenesis is characterized with invasion of the erythrocytes with the release of free haem and hydrogen peroxide (H₂O₂) (Somsak et al. 2016). Accumulation of malaria-induced free radicals has been associated with loss or reduction of functions in various tissues of the body (Becker et al. 2004), brain and skeletal muscles inclusive. Cerebral malaria (CM) ranks as the most dangerous, with the highest fatality of all manifestations of severe malaria (Mavondo et al. 2016). A pathophysiological symptom associated with CM is the convulsive status epileptics, characterized by constant seizure crisis and coma (Birbeck et al. 2010). CM has been found to be as a result of sequestration of parasitized red blood cells and adherence to the endothelium by parasite derived proteins located on red cell surface, leading to blood flow obstruction to distinct areas of the central nervous system (Van der Heyde et al. 2006). Cerebral malaria has been implicated in cognitive, behavioral and motor dysfunctions (Oliveira et al. 2017). These dysfunctions can also persist long term, even after treatment (Oluwayemi et al. 1999; Reis et al. 2010). The positive effects of antioxidants in reducing the manifestations of severe malaria have been reported (Reis et al. 2010), even in specific cases like cerebral malaria.

The antimalarial and antioxidant activities of *Phyllanthus amarus* has been reported (Komlaga et al. 2015; Oyewole et al; 2013), but reports of its ability in suppressing the incidence of cerebral malaria is scarce. Therefore, this study assesses indicators of cerebral malaria (parasite count and chemosuppression), haematological indices (packed cell volume and haemoglobin), preventive antioxidants (ferritin and myoglobin) status, free radical scavengers and nitric oxide concentrations in blood and brain tissues of *Plasmodium berghei* infected mice and the impact of *Phyllanthus amarus* phytochemical extracts on these indices in infected mice. The antiplasmodial activity of *Phyllanthus amarus* is further confirmed by our study. Parasitaemia and parasite count in blood and brain tissues of infected mice decreased with administration of phytochemical extracts of *Phyllanthus amarus*. Greatest antiplasmodial activity was demonstrated by the alkaloid phytochemical. This is consistent with other studies attributing the antiplasmodial activity of *P. amarus* and antimalarial plants to their alkaloid content (Uzuegbu et al. 2020; Bapna et al. 2014).

Glucose abnormalities (hyperglycemia, hypoglycemia and hyperinsulinemia) have been implicated in severe malaria (Gildas et al. 2017; Mavondo et al. 2019). Hyperglycemia is more common than hypoglycemia, but only hypoglycemia is associated with an increased risk of death (Gildas et al. 2017). Blood glucose is increased in uncomplicated malaria, but as disease progresses, risk of



hypoglycemia and insulin resistance increases (Binh et al. 1997), suggesting that glucose abnormalities are dependent on parasite density. Hypoglycemia in blood has been reported to be strongly correlated to the incidence of cerebral malaria. (Kochar et al. 1998). From our study, hyperglycemia was recorded in the brain tissues and severe hypoglycemia was observed in blood of malaria infected mice. Hyperglycemia could be as a result of decreased glucose uptake and oxidation by the brain due to sequestration of parasitized RBCs, platelets and leukocytes in the brain vessels. This accumulation, causes endothelium damage, which leads to the break down of the blood brain barrier and disruption of osmotic equilibrium between brain and plasma (Cox et al. 2007). Also, malaria causes release of hormones and cytokines, increasing hepatic glucose production and insulin resistance (Mavondo et al. 2016). Hypoglycemia in blood is caused by the high demand of parasitized RBCs for glucose from hosts RBCs (Ghosh et al, 2018) as intraerythrocytic *Plasmodium* lack gluconeogenic enzyme, fructose 1,6 biphosphate and glycogenolytic enzymes (Srivastava et al. 2016).

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Wang et al. (2018) showed that a glycolytic inhibitor, 2-deoxy glucose (2DG), prevented the development of cerebral malaria. Although, 2DG did not reduce inflammation or anaemia, it improved tolerance by affecting homeostasis and prevented hemorrhage in experimental mice, increasing survival time. However, mice succumbed to anaemia. Therefore, agents that could inhibit glycolysis could help in preventing cerebral malaria. Our study reports that phytochemical extracts of *P. amarus*, saponin, glycosides and alkaloids, reduced the incidence of hyperglycemia in brain tissue and subsequently, hypoglycemia in blood was alleviated by the alkaloid, tannin and saponin phytochemicals. This suggests that the alkaloid and saponin phytochemicals of *P. amarus* strongly reduced the severe malaria manifestations by either inhibiting glycolysis or as part of its antiplasmodial action.

Haematological parameters, haemoglobin (Hb) and packed cell volume (PCV), were decreased following malarial infection, suggesting anaemia which have been confirmed by other researchers (Adamu and Jigam, 2019; Al-Salahy et al. 2016). Oxidative stress plays an important role in the development of malarial anaemia (Chandra et al. 2006), thus, making anaemia a reliable predictor of the severity of disease. Decreased PCV can be related to increased haemolysis of infected and non infected RBCs in malarial infection (Akinosoglou et al. 2012). Other mechanisms such as impaired erythropoiesis (Abdalla, 1990), iron sequestration and iron deficiency can contribute to anaemia (Brabin, 1992). Severe malaria has repeatedly been reported to cause induction of anaemia through CD8⁺ T cell-dependent plasmodial clearance and red cells removal in the spleen (Noone et al. 2013). All phytochemical extracts of *P. amarus* ameliorated the adverse effect of malarial infection by increasing Hb and PCV concentrations in experimental mice. The action of *P. amarus* in this study can be related to other studies that reported increased concentrations of Hb (Nwakpa et al. 2014) and PCV (Kolawole et al. 2019) in experimental mice.

Malaria, being an inflammatory disease, is characterized by increased ferritin concentrations (Verhoef et al. 2001). Ferritin estimation without examination for malarial parasitaemia is not reliable as ferritin concentrations will increase in all inflammatory conditions (Odunukwe et al. 2000). However, serum ferritin levels have been shown to increase with increasing malaria density (Abdullahi et al. 2021; Odunukwe et al. 2000). Some data suggest that ferritin concentration may not be affected in patients with malarial parasitaemia levels of <1000 parasites/ μ L (0.02%) (Northrop-Clewes, 2008). Serum ferritin increases as iron accumulates in the iron store and decreases as storage iron levels drop (Abdullahi et al. 2021). Treatment of infected experimental mice with alkaloid and anthraquinone phytochemicals of *P. amarus* reduced serum ferritin levels. This further



confirms the antiplasmodial activity of the alkaloids of this plant. However, estimation of serum ferritin levels in malarial infection may not accurately reflect body iron stores.

Increased myoglobin, a marker of cardiac and striated muscles, concentrations was also found in malarial infected mice in our study, suggesting rhabdomyolysis. Bachmann et al. (2014) reported increased myoglobin in children presenting cerebral malaria and suggests that the vascular smooth-muscle cell-layer and extra-vascular striated muscles appear to be an important site for sequestration of parasitized RBCs. Elevated myoglobin concentrations in cerebral malaria has also been confirmed by other researchers (Ehrhardt et al. 2004; Yong et al., 2012; Olaniyan and Babatunde, 2016). Excess myoglobin in blood circulation could lead to kidney failure if filtrated by kidneys (Bachmann et al. 2014). Yong et al. (2012) reported acute kidney infection (AKI) in cerebral malaria and related it to myoglobin-induced renal vasoconstrictions, direct nephrotoxicity (as myoglobin is both directly and indirectly toxic to kidney) and sequestered infected erythrocytes in renal muscles. Myoglobinuria have been found in the urinalysis of patients with severe malaria (Mishra et al. 2010). Hence, the measurement of serum and urine myoglobin concentrations confirm the rationale for the determinant of the severity of the disease in our study. Myoglobin concentrations in mice post treatment with phytochemical extracts of *P. amarus* were greatly reduced, but, not as concentrations in uninfected mice. Further confirming its antiplasmodial activity, the alkaloid phytochemical demonstrated the most activity.

The use of antioxidants in reducing the risk of severe malaria in experimental animals have been documented. The efficacies of antioxidants are usually associated with their ability to inhibit oxidative damage by scavenging free radicals. Antioxidants have been studied for their antiplasmodial activity. Reis et al. (2010) study on antioxidant therapy with N-acetyl cysteine and desferroxamine as an additive to chloroquine, prevented insistent cognitive damage in cerebral malaria. Lipid peroxidation characterized with increased levels of MDA (Mozafari et al. 2016) was found in brain tissues of malaria infected mice. Ries *et al.* (2010) confirmed increased MDA in the brain of mice with cerebral malaria. Antioxidant therapy with N-acetyl cysteine and desferroxamine, as an additive to chloroquine, prevented the cognitive impairment, suggesting antioxidant therapy as a successful strategy to control long-lasting consequences of cerebral malaria. Our study shows that the flavonoid phytochemical extract of *P. amarus* reversed the effect of malaria on MDA levels. This suggests that the flavonoid phytochemical is an inhibitor of lipid peroxidation. Mallaiah et al. (2015) and Ogunmoyole et al.(2020) have reported the inhibitory lipid peroxidation effects of the extracts of *P. amarus* in patients infected with malaria. Londheet al. (2008) showed that the polyphenol constituents of *P. amarus* protects rat liver mitochondrial membrane against oxidative damage. Confirming this activity, Nikam et al. (2011)also reported that extracts of *P. amarus* inhibited lipid peroxidation in ethanol-induced liver damage and associated this activity with polyphenol constituents of *P. amarus*.

The main antioxidant in the brain, reduced glutathione (GSH), is greatly decreased in cerebral malaria (Oliveira et al. 2017). Increased parasitaemia has been found to be accompanied with a decrease in the activities of other enzymes of the glutathione system (Sarin et al. 1993). Prabhu et al. (2021), showed the 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 in *P. berghei* infectedmice. Reduced GPx and GSH levels in *P. falciparum* infected children has also been reported (Oluba, 2019). From our study, all phytochemical extracts of *P. amarus* produced increased concentrations of GSH, GPx and GST in brain tissues.



The balance of superoxide dismutase and catalase is essential to combat superoxide anion and peroxides generated in oxidative stress (Azubuike et al. 2018). The reduced activities of CAT and SOD in brain of infected mice, as observed in this study is a clear indication of oxidation by malarial infection. Francischetti et al. (2014) conducted a study on the administration of tempol (an SOD simulator) on cerebral malaria and found that tempol inhibits tissue factor expression and attenuates dendritic cell function, highlighting its protectiveness in cerebral malaria. They also reported its role in decreasing permeability of the blood brain barrier in CM. This suggests that antioxidants directed at stimulating SOD activity, reduces the risk and severity of CM. Also, Thumwood et al. (1989) reports that administration of a combination of a of catalase and superoxide dismutase coupled to polyethylene glycol partially increased survival time in cerebral malaria. Therefore, SOD and CAT stimulators can slow down the progress of cerebral malaria. All phytochemical extracts of *P. amarus* in our study, increased the concentration of SOD and CAT in brain tissues, suggesting that *P. amarus* stimulated the production of these enzymes.

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Myeloperoxidase (MPO), secreted by activated neutrophils as an inflammatory response (Mohammed et al. 2013), has been reported to lessen adaptive immune response in malarial infection, hence reducing rate of parasite clearance (Theeß et al. 2016). This confirms its role in the pathogenesis and severity of the *Plasmodium* infection. This is also confirmed by our study. However, administration of *P. amarus* phytochemical extracts did not sufficiently reduced MPO concentrations in brain tissues.

Excess superoxide as a result of reduced SOD concentration will react with nitric oxide (NO) and, therefore, will reduce NO bioavailability causing vasoconstriction, loss of anti-inflammatory and anti-adhesive functions of NO (Finkel, 2011). Elevated serum concentration of myoglobin, as reported by our study, has also been reported to reduce NO bioactivity (Prabhakar et al. 2014). Vasoconstrictions could cause acute tubular necrosis leading to acute renal failure in CM (Prabhakar et al. 2014). Patients with higher blood levels of NO have increased protection against severe malaria (Crammer et al. 2005). This can be related to reduced nitric oxide synthase activity (Halaris and Plietz, 2007) by haemozoin interactions with L-arginine (a NO precursor) reducing its availability for nitric oxide synthase, hence, reducing nitric oxide production and bioavailability (Corbett et al. 2018). After administration of *P. amarus*, increased nitric oxide was found in brain tissues of test mice in our study, suggesting stimulation of nitric oxide synthase or its action on increased superoxide dismutase activity. Either way, bioavailability of NO in brain tissues increased. Cabrales et al. (2011) reported that NO in conjugation with antimalarials in experimental mice enhanced protection from cerebral malaria by improved brain microcirculatory hemodynamics and decreased vascular pathology.

Conclusions

Our study indicates that infection of experimental mice with *P. berghei* (NK65) malarial parasite, induced cerebral malaria (CM) within nine days post infection. Such induced CM was observed to be associated with anaemia, increased brain glucose, but reduced in blood, increased oxidative assault in brain and reduced cerebral NO concentration.

However, treatment of the induced malarial infection with standard drug (Lonart®DS) and phytochemical (alkaloid, flavonoid, tannin, saponin, anthraquinone and glycoside) extracts of *P. amarus* ameliorated the biochemical evidence of CM. Nonetheless, the alkaloid extract demonstrated the greatest significant activity in reducing cerebral parasite counts, anaemia and NO which has been reported (Corbett et al. 2018) to bear reverse relationship with haemozoin, a



malaria pigment obtained from the metabolism of haem. But, its antioxidant activity was not superior compared with the flavonoid extract.

Our investigation, therefore, identifies alkaloid extract of *P. amarus* as capable of significantly suppressing both brain and blood malarial parasites, and also, ameliorating the associated derangements. Judging with the brain NO concentration, we speculate that alkaloids of *P. amarus* inhibit parasite multiplication by possibly interfering with haem polymerization to haemozoin, a powerful antiplasmodial mechanism. The extract (alkaloid) also protected brain cells from the parasites'-induced oxidative damage by enhancing antioxidant defense via induction of free radical scavenging enzymes and preventive antioxidants.

Thus, the alkaloid and flavonoid compounds of *P. amarus* need to be isolated for synergistic study.

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