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Antimalarial and antioxidant properties of *Vernonia amygdalina* ethanol leaf extract in mice infected with rodent malaria parasites

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Abstract
This study investigated antimalarial and antioxidant activity of *Vernonia amygdalina* ethanol leaf extract on mice infected with plasmodium berghei. LD_{50} value of extract was greater than 3200mg/kg/bt. DPPH and hydrogen peroxide activity showed concentration dependent increase. Significant ($p<0.001$) suppression of parasite was 63.7% and 77.0% at 150mg/kg and 300mg/kg respectively was recorded in the suppressive test while 75.8% suppression of parasitaemia was recorded at 300mg/kg of extract in established malaria infection and prolonged their survival time up to 28 days compared to negative control which died within 13 days. Increase in reduced glutathione and decrease in malondialdehyde activity indicate reduction in oxidative stress while decrease in catalase activity implies oxidative stress. The extract increased packed cell volume and red blood cell count of mice. *V. amygdalina* leaf extract possesses antioxidant and potent antimalarial potentials which can be harnessed for the production of antimalarial pharmaceuticals and can be further accessed for treatment of anemia.

Keywords: *V. amygdalina*, parasitaemia, antioxidant, antimalarial, P. berghei

Introduction
The malaria parasite, belonging to the genus "*Plasmodium*'' is a major cause of severe illness in both humans and animals. Infections by this parasite lead to the life-threatening disease known as malaria. According to the World Health Organization (WHO), there were an estimated 241 million malaria cases worldwide in 2020, resulting in approximately 627,000 death [1]. Children under the age of five and pregnant women are especially at risk of developing severe malaria. In a previous report from 2015, the WHO noted 214 million malaria cases and 438,000 deaths, with around 70% of these fatalities occurring in children under five[2] The high mortality rates associated with malaria are primarily due to the parasite's resistance to commonly used antimalarial medications [3].

Artemisinin-based Combination Therapies (ACTs) are recognized as the first-line of treatment for uncomplicated malaria caused by *P. falciparum*. Combining ACTs with other antimalarial drugs has been suggested to enhance treatment efficacy and minimize the likelihood of resistance[4]. This method has gained widespread acceptance as the global standard for treating malaria, due to its proven effectiveness and high rates of successful treatment outcomes. Derivatives of artemisinin, such as artesunate, artemether, and dihydroartemisinin, originate from the plant 'Artemisia annua' These drugs are currently some of the most effective options available, showing significantly faster parasite clearance compared to other treatments [3].

Antioxidants are compounds that counteract free radicals and prevent damage caused by reactive oxygen species (ROS) [5]. They play important role in the management of malaria as a result of their potential effects on oxidative stress which a major feature of malaria disease. Antioxidant prevents oxidative stress by neutralizing ROS and repairing oxidative damage. Antioxidants can be endogenous, naturally produced by the body and include enzymes like superoxide dismutase, catalase and gluthione perioxidase and also non-enzymatic antioxidants like gluthione (GSH) or exogenous, that is those obtained from diets and supplements which include vitamins and polyphenols.

Living organisms have evolved a complex system of internal antioxidant defenses to either prevent the overproduction of free radicals or to minimize the damage they can cause [6]. Moreover, both internally produced and externally sourced antioxidants in the human body play a role in reducing the harmful effects of oxidative stress that often accompanies various diseases [7].

Antioxidants are essential molecules that protect cells from damage caused by free radicals-unstable molecules with unpaired electrons. These antioxidants function by acting as reducing agents, donating electrons to stabilize or neutralize free radicals before they can cause harm to cells. This protective action makes antioxidants crucial in managing and treating different forms of cellular degeneration [8].

Vernonia amygdalina, commonly known as bitter leaf, belongs to the Asteraceae family and is commonly found across tropical Africa. It is often grown as a food supplement in regions of West Africa, including Nigeria [9]. This plant typically appears as a small tree that reaches heights between 2 to 5 meters. It features dark green, glossy leaves, along with small flowers that are either white or purple. As a perennial plant, it can grow to a height ranging from about 1 to 6 meters [10].

***Vernonia amygdalina* plant (Source: Wikipedia)**

Vernonia amygdalina Del, widely referred to as bitter leaf, is known locally as 'Shuwaka' in the Hausa language and 'Ewuro' in Yoruba. This plant is often found growing abundantly along drainage channels, in natural forests, and in home or commercial plantations, especially thriving during the rainy season.

The leaves, stems, and roots of *V. amygdalina* are well-regarded for their medicinal properties. There have been reports indicating that both aqueous and ethanol extracts derived from the stem, bark, and leaves of this plant have traditionally been used as a purgative, an antimalarial remedy, and a treatment for eczema[11]. Therefore this study aims to evaluate antimalarial and antioxidant potentials of *Vernonia amygdalina* leaf extract and ascertain its efficacy as a possible treatment option for malaria using mice infected with *Plasmodium berghi*.

Methodology

Chemicals and Reagents

Chemicals and reagents utilized in this study were of analytical grade and were employed without any additional purification. These materials were sourced from Joechem Ventures, located in Port Harcourt, Rivers State, Nigeria.

Ethical Implications

All experimental procedures carried out in this study adhered strictly to the guidelines outlined in the "Guide to the Care and Use of Animals in Research and Teaching" [12]. The Department of Pharmacology at the University of Port Harcourt, Rivers State, thoroughly reviewed and granted approval for all experiments.

Leaf Sample Collection

Leaves of *Vernonia amygdalina* were harvested in September 2021 from Obodoukwu, located in Ideato Local Government Area of Imo State, Nigeria. A plant taxonomist from the Department of Biological Sciences at the Federal University of Technology, Owerri, Nigeria, verified their identity, and a voucher specimen with number 297/2021 was recorded.

Plant Extraction

The *Vernonia amygdalina* leaves were thoroughly rinsed with clean water and left to air-dry at room temperature for about 7 to 10 days. Once dried, the leaves were ground into a fine powder using an electric blender. A sample weighing 400 grams was then soaked in 80% ethanol for 72 hours, with continuous agitation using a GFL shaker. The mixture was subsequently filtered through filter paper, and the resulting filtrate was evaporated to dryness under reduced pressure at 40 °C using a rotary evaporator. The final dried extract was stored at a low temperature until it was needed for further use.

Identification of parasites

The *Plasmodium berghei* strain used in this study was sourced from a donor mouse infected with the parasite, which was housed at the Animal Facility Centre of the Faculty of Veterinary Medicine at the University of Nigeria, Nsukka. Blood samples for infecting the mice were collected via cardiac puncture from the donor mouse which had a parasitemia level of 40%. The parasitemia of the donor mouse was assessed through microbiological examination of the blood sample. This involved preparing a blood smear on a glass slide, staining it with appropriate dyes, performing a microscopic examination using immersion oil, and calculating the parasitemia level [13].

Experimental Animals

A total of Eighty-five (85) adult mice, comprising both sexes and weighing between 20 and 32 grams were acquired from the Laboratory Animal House of the Pharmacology Department at the University of Port Harcourt, Rivers State. The mice were acclimatized for approximately two weeks under standard environmental conditions, with unrestricted access to commercial rat pellets and water. Out of the total, 35 mice were designated for the acute toxicity test, while 25 were used in the suppressive antimalarial test, and another 25 were utilized for the curative/Rane test. The use of these animals adhered to the guidelines and recommendations set forth by the ethics committee regarding animal research at the University of Port Harcourt.

Antimalarial Drug

Artesunate tablet 100mg was dissolved in phosphate buffer saline (PBS) to final dose of 10mg/kg body weight and used for inoculation/treatment.

Phytochemical test

Phytochemical components of *V. amygdalina* leaf extract were ascertained using standard procedures [14, 15] to ascertain its active components.

Total phenols determination

The most common method for determining total phenol

content in leaf extract is Folin-Ciocalteu assay. The total phenolics content of *V. amygdalina* leaf extract was determined using Folin Ciocalteu reagent using the modified method [16].

0.5 ml of *V. amygdalina* leaf extract was mixed with 10% Folin Ciocalteu reagent solution diluted in 2.5ml of distilled water. 2.5ml of sodium carbonate solution was added to the mixture and vortexed for 15seconds and incubated at room temperature for 30 mins for colour development. The absorbance of the samples was measured at 765nm using spectrophotometer. Total phenolic content of the extract was expressed as mg/g tannic acid equivalent from a calibration curve using the equation: Y=0.1216x Where x was the absorbance and Y was the tannic acid equivalent (mg/g).

Total flavonoids determination

The total flavonoids content of the extract was estimated using standard method [17] based on the formation of a complex flavonoid-aluminium. 0.5 ml of 2% $AICI_3$ in ethanol solution was added to 0.5ml of extract solution. After few minutes of incubation at the room temperature, the absorbance was measured at 420nm using UV-VIS spectrophotometer.

A yellow colour indicated the presence of flavonoids. All determinations were calculated using calibration curve. Total flavonoid content (mg/g) = (Absorbance X dilution factor) /Slope of calibration curve.

Lethal Dose (LD_{50}) Studies

Lethal dose assessment of *V. amygdalina* leaf extract was determined using standard test and procedures[18]. Thirty five (35) mice starved for 16 hours were randomly divided into 7 (seven) groups of 5 (five) mice each. Graded doses of the extract (100, 200, 400, 800, 1600 and 3200mg/kg/b.wt) were separately administered to mice in each test group using 1ml syringe and control group were administered with normal saline (0.3 ml/kg) only. All animals were then allowed free access to feed and clean water and were observed for a period of 48hrs for signs of acute toxicity such as aggression, lethargy, difficulty in breathing, paralysis, lesions, uncoordinated movement and other toxicity signs including mortality.

Parasite Inoculation

Suppressive test

Suppressive effect of the extract in early malaria infection was ascertained with Peter's 4-day suppressive test [19]. Twenty-five albino mice were used and twenty (20) of them were administered with 0.2 mL suspension of parasitized erythrocyte with the aid of a syringe intraperitoneally while 5 (five) mice were left unparasitized. After 3 hours, mice were divided into five groups of five mice each.

Group 1-Mice not parasitized and not treated (Positive control)

Group 2-Mice were parasitized and given 2ml of phosphate buffer saline (PBS) as negative control

Group 3-Mice were parasitized and were administered with 150mg/kg/b.wt of *V. amygdalina* leaf extract

Group 4-Mice were parasitized and administered with 300 mg/kg/b.wt of *V. amygdalina* leaf extract

Group 5-Mice were administered with 10mg of Artesunate antimalarial drug.

"Treatment was administered for four consecutive days. On the fifth day, the parasitemia of each mouse was determined. Tail blood samples were collected, thin blood smears were prepared, and these smears were fixed with methanol. The fixed smears were stained with Giemsa and examined microscopically at 100x magnification. To calculate the percentage suppression of parasitemia, the parasitemia levels in treated mice were compared to those in infected but untreated control mice [20].

$$\% \text{ Inhibition} = \frac{\text{Parasitamia in control - Parasitaemia in treated}}{\text{Parasitaemia in control}} \times 100$$

Curative Test

A total of twenty-five (25) Swiss albino mice were utilized for this experiment. On the initial day (D0), the mice received an intraperitoneal injection of 0.2 ml suspensions containing parasitized red blood cells. After 72 hours, the mice were sorted into five (5) groups, each consisting of five mice.

Group 1-Mice not parasitized and not treated (Positive control)

Group 2-Mice were parasitized and given 2ml of phosphate buffer saline (PBS) as negative control

Group 3-Mice were parasitized and were administered with 150mg/kg/b.wt of *V. amygdalina* leaf extract

Group 4-Mice were parasitized and administered with 300 mg/kg/b.wt of *V. amygdalina* leaf extract

Group 5-Mice were administered with 10mg of Artesunate antimalarial drug.

Phosphate-buffered saline, extract, and drug were administered orally once per-day over a period of five days. For four consecutive days, thin blood smears were taken from the tails of each mouse to monitor parasitemia levels, percentage inhibition, red blood cell count, and packed cell volume (PCV) throughout the study. The average survival time for each group was determined using a standard method outlined in reference [21], and the percentage of parasitemia was calculated using the formula.

$$\text{Percentage Parasitaemia (\%)} = \frac{\text{Number of infected red blood cells (RBCs)}}{\text{Total number of red blood cells}} \times 100$$

Estimation of Mean Survival Time (MST) of Mice

The duration from the inoculation of the mice until their death was tracked and documented to determine the mortality rate for each group. The mean survival time (MST) was calculated using the following formula [22]:

$$\text{Mean Survival Time (MST)} = \frac{\text{Sum of survival time of mice in a group (days)}}{\text{Total number of mice in that group}}$$

Determination of Packed Cell Volume (PCV)

In established malaria infection (Rane Test), packed cell volume (PCV) of each mouse was determined before infection, 72 hours after infection and 4 days post-treatment. Blood were collected from the tail of individual mouse into heparinized hematocrit tubes, sealed with low flame, and placed in a hematocrit centrifuge at 10,000 rpm for 5 min. PCV was read using hematocrit reader according to the following formula [23]:

$$\text{PCV} = \frac{\text{Volume of packed red blood cells}}{\text{Total volume of blood}} \times 100$$

Estimation of Radical scavenging activity of *V. amygdalina* leaf extract

The antioxidant activity of the *V. amygdalina* extract was evaluated using a modified method [24] to ascertain its radical scavenging activity. Solutions of varying concentrations (25, 50, 100, 200, 250, and 500 mg/ml) were prepared and mixed with 0.2 mM DPPH solution in methanol in a '1:1' ratio. The mixtures were incubated in the dark for 30 minutes, and their absorbance was measured at 517 nm using a UV spectrophotometer. The tests were conducted in triplicate, and vitamin C served as a control. A blank containing 0.5ml of 0.2mM DPPH and 2ml methanol was prepared and treated as the test standard.

(2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = [(AO-A1)/ (AO)] x 100

Where AO = Absorbance of DPPH solution without extract (control), A1 = Absorbance of DPPH solution with extract.

Estimation of Hydrogen peroxide scavenging activities of *V. amygdalina* leaf extracts

The hydrogen peroxide scavenging activity of the extract was evaluated following a standard procedure. A 43 mM hydrogen peroxide solution was prepared in a phosphate buffer at pH 7.4. Varying concentrations of the extracts (25, 50, 100, 200, 250, and 500 µg/ml) were combined with the hydrogen peroxide solution in equal volumes ('1:1', v/v) and left to incubate at room temperature for 10-15 minutes. The absorbance of the resulting mixtures was recorded at 230 nm using a spectrophotometer. This process was repeated for each concentration of the extract, and the hydrogen peroxide scavenging activity was calculated using the formula:

% H_2O_2 scavenging activity (%) = [(A0-A1)/A0] x 100

Where AO = Absorbance of hydrogen peroxide solution without plant extract (control)

A1 = Absorbance of the mixture containing plant extract and hydrogen peroxide

Biochemical Assay

On the 10th day, three mice from each group were humanely euthanized following the approved protocol. Liver samples were collected, rinsed with phosphate-buffered saline, and then homogenized. The homogenate was centrifuged at 10,000 rpm for 10-15 minutes to separate the supernatant from the pellet. The supernatant was kept at 4 °C until it was used to assess antioxidant enzyme levels, including malondialdehyde [26] (MDA), Glutathione [27] (GSH) and catalase [28] (CAT).These measurements were carried out using a spectrophotometer.

Statistical analysis

The data were presented as mean ± standard error of mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Post-hoc multiple comparison test using the Turkey method for paired samples. A p-value of less than 0.001 was considered statistically significant.

Results

Phytochemical Composition: Initial phytochemical analysis of *Vernonia amygdalina* leaf extract revealed the presence of various bioactive compounds, including saponins, flavonoids, tannins, alkaloids, cardiac glycosides, steroids, phenols, and terpenoids. The quantitative assessment showed a particularly high concentration of phenolic compounds (46.9±0.96), along with significant levels of saponins, alkaloids, and other bioactive components (Table1).

Table 1: Phytochemical composition of *vernonia amygdalina* ethanol leaf extract

Phytochemicals	Contents	Compositions (Mg/g)
Flavonoid	+	3.16±0.03
Tannins	+	3.56 ± 0.39
Alkaloids	+	4.82±0.64
Saponins	+	12.19±0.12
Cardiac glycosides	+	9.5±2.89
Steroids	+	4.50±0.32
Phenols	+	4.40±0.31
Tepernoid	+	1.82±0.06
Phlobatanins	_	_
	Total Flavonoids	4.62±0.50
	Total Phenolics	46.9±0.96

Key: + presence of phytochemical, - absence of phytochemical, Data are expressed as mean±SEM, based on a sample size of three (N=3)

Lethal Dose (LD_{50}) Assessment Results: The lethal dose evaluation of *Vernonia amygdalina* ethanol leaf extract demonstrated its non-toxic effect on healthy mice at doses of 100 mg/kg, 200 mg/kg, 400 mg/kg, 800 mg/kg, 1600 mg/kg, and 3200 mg/kg body weight, with no deaths observed. However, mice treated with the highest dose of 3200 mg/kg exhibited labored breathing and restlessness for several hours before returning to normal behavior. The other experimental groups displayed consistent normal behavior for more than 72 hours.

DPPH free radical scavenging activity of *vernonia amygdalina* extract: The extract of *Vernonia amygdalina* demonstrated a significantly higher DPPH free radical scavenging activity ($p<0.001$) compared to the standard ascorbic acid at the same concentrations (25µg/ml, 50µg/ml, 10µg/ml, 200µg/ml, 250µg/ml, and 50µg/ml). Specifically, the DPPH radical scavenging activity of the extract at doses of 500 µg/ml and 25 µg/ml was 68% and 18%, respectively, whereas ascorbic acid showed activities of 92% and 30% at the same concentrations as shown in Figure 1.

Hydrogen peroxide scavenging activity of *vernonia amygdalina* leaf extract: *V. amygdalina* leaf extract showed significant ($p<0.001$) concentration dependent hydrogen peroxide scavenging effect. The extract scavenged 49.6% and 7% at 500 µg/mL and 25 µg/ml concentrations respectively while ascorbic acid which was the standard scavenged 91% and 32% at the same concentrations (Figure 2).

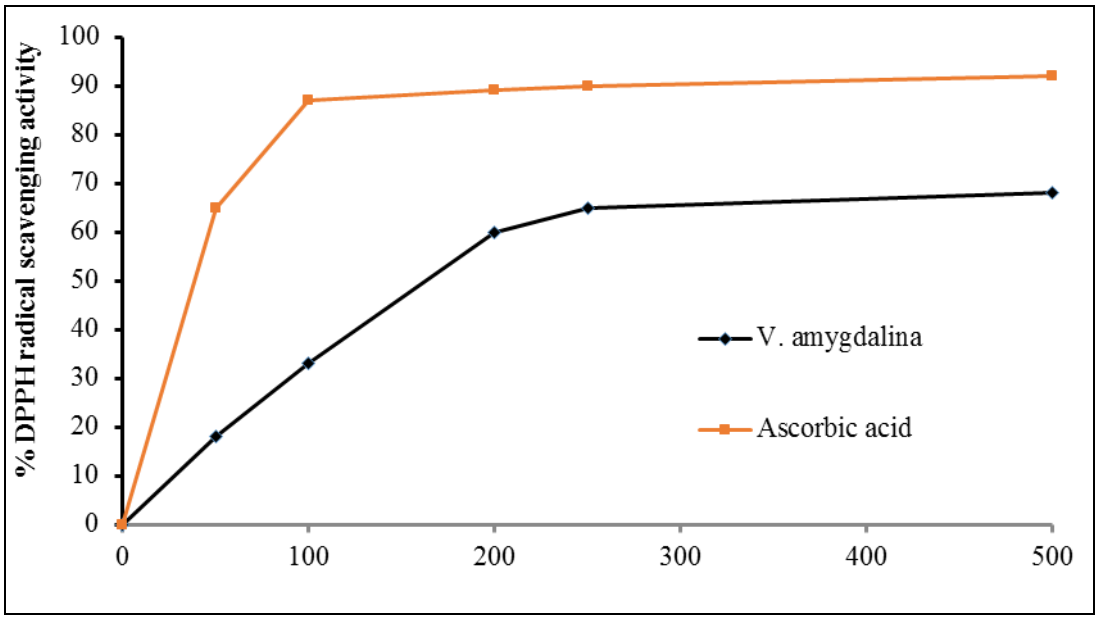

Fig 1: 1,1-Diphenyl-2-picryl hydrazyl (DPPH) activity of *V. amygdalina* ethanol leaf extract.

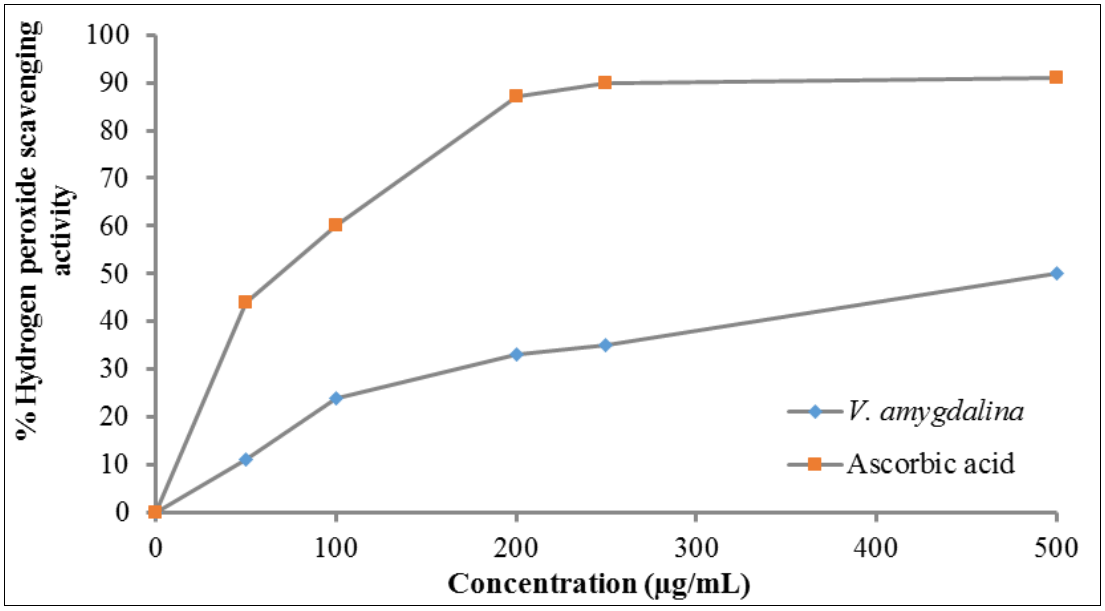

Fig 2: Hydrogen peroxide scavenging activity of *V. amygdalina* leaf extract

Effect of Extract in Early Malaria Infection (Suppressive Test)

Ethanol leaf extract of *Vernonia amygdalina* showed a dose-dependent reduction in parasitemia, achieving a 77.0% decrease at a dosage of 300 mg/kg and a 63.7% decrease at 150 mg/kg body weight. In contrast, the group treated with Artesunate exhibited an 81.7% reduction, surpassing the effects observed in the extract-treated groups. (Table 2).

Table 2: Effect of *vernonia amygdalina* extract in early malaria infection

Treatment Drug/extract	Dose	Parasite count treatment	% Inhibition
Normal saline	2ml/kg	58.36±0.43	0
V. amygdalina	150mg/kg/b.wt	20.95±0.39*	63.7%
V. amygdalina	300mg/kg/b.wt	13.80±0.39*	77.0%
Artesunate	10mg/kg/b.wt	10.28±0.19*	81.7%

Significantly different from the saline control group at $p<0.001$; N = 5.
Data are presented as Mean ± SEM.

Effect of the extract in established malaria infection (Curative/Rane test)

The *Vernonia amygdalina* extract showed a significant reduction in parasitemia ($p<0.001$) from day 5 to day 7 of treatment, with parasite inhibition rates of 66.2% at 150 mg/kg and 75.8% at 300 mg/kg. Mice treated with the extract survived for more than 27 days, comparable to those in the Artesunate group, which achieved a 78.4% inhibition rate at a dose of 10 mg/kg.

Table 3: Impact of *vernonia amygdalina* ethanol leaf extract in established malaria infection

Treatments	Dose	Parasite count day 5	Parasite count day 7	Mean survival time in days	% Inhibition
Normal saline	2ml/kg	58.36 + 0.43	64.52+ 0.74	13+ 0.63	0
V. amygdalina	150mg/kg/b.wt	29.29 + 0.72*	19.20+ 0.67*	25+ 0.49**	66.2%
V. amygdalina	300mg/kg/b.wt	31.35 + 0.92*	14.89+ 0.58*	27+ 0.24**	75.8%
Artesunate	10mg/kg/b.wt	31.46 + 0.41*	13.59+ 0.41*	27+ 0.79**	78.4%

$p<0.001$ indicates a significant decrease compared to the normal saline control group. **$p<0.001$ indicates a significant increase compared to the normal saline control group. Sample size (N) = 5. Data are presented as Mean±SEM

Biochemical activities of *V. amygdalina* extract

Malondialdehyde: The extract caused a significant reduction ($p<0.001$) in malondialdehyde activity at both 150 mg/kg and 300 mg/kg doses, as well as in the positive control group treated with 10 mg/kg of Artesunate. In contrast, the negative control group given normal saline showed a marked increase in malondialdehyde activity compared to groups treated with *Vernonia amygdalina* extract.

Catalase

The extract led to a significant reduction ($p<0.001$) in serum catalase activity at both 150 mg/kg and 300 mg/kg doses. A notable difference ($p<0.001$) in catalase activity was observed in the uninfected group (14.53±1.08) compared to extract-treated groups at 150 mg/kg (9.56±0.22) and 300 mg/kg (10.30±0.48), as well as the untreated group (10.20±0.36).

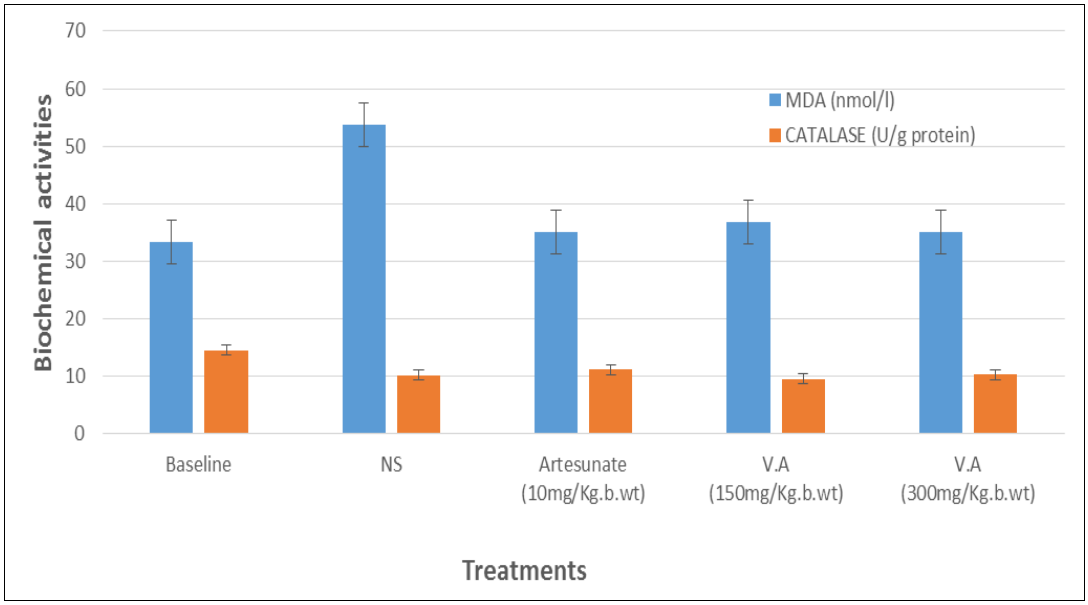

Fig 3: Impact of *vernonia amygdalina* leaf extract on Malondialdehyde (MDA) and Catalase Levels

Reduced glutathione (GSH): Administration of *Vernonia amygdalina* ethanol leaf extract at doses of 150 mg/kg and 300 mg/kg resulted in notable increase in glutathione (GSH) activity, in contrast to the untreated group, which showed a statistically significant decrease in GSH activity ($p<0.001$). Figure 4.

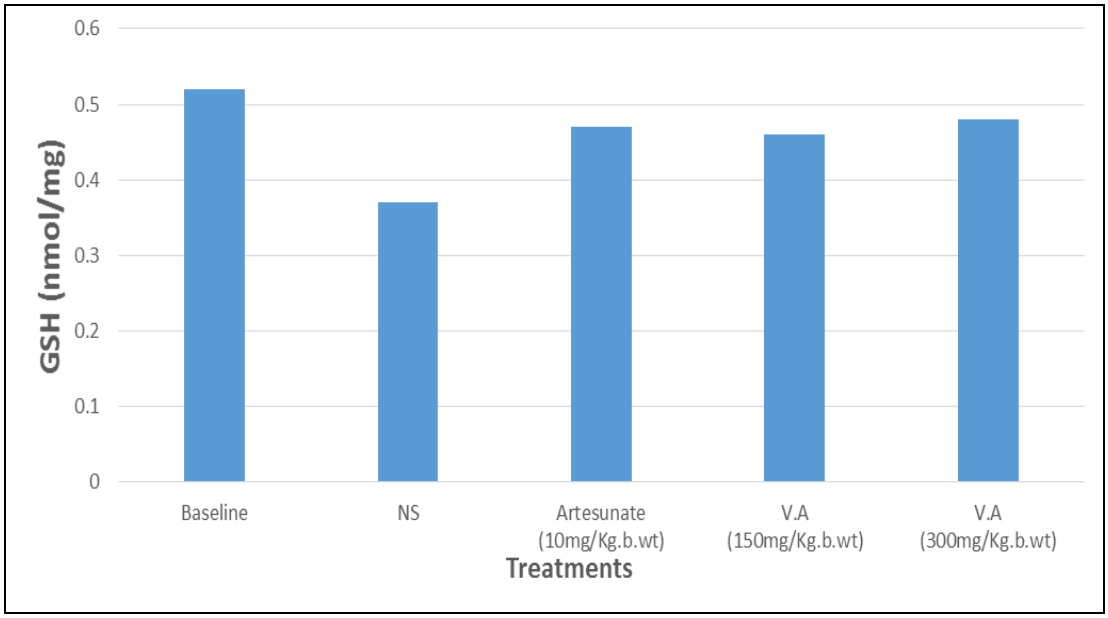

Fig 4: Impact of *V. amygdalina* leaf extract on reduced glutathione

Hematological Activity

Packed cell volume (PCV): Before infection, no significant difference in packed cell volume (PCV) was observed across all groups. However, after 72 hours of inoculation, the extract increased PCV levels in the 150 mg/kg and 300 mg/kg treatment groups. A marked increase in PCV ($p<0.001$) was evident after 4-days of treatment compared to the negative control group, which showed a steady decline in PCV. Similarly, the positive control group treated with 10 mg/kg of Artesunate also demonstrated an increase in PCV.

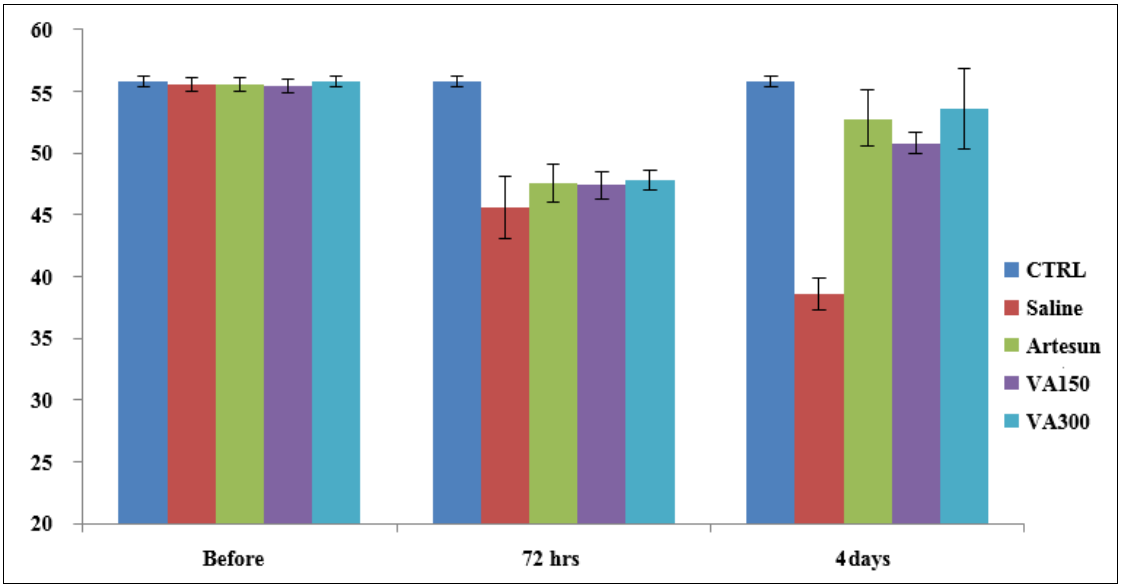

Fig 5: Average packed cell volume before infection, 72 hours post-infection, and after 4 days of treatment

Red blood cell (RBC) counts (10^6/L)

The *Vernonia amygdalina* leaf extract led to significant increase ($p<0.001$) in red blood cell (RBC) count at doses of 150 mg/kg and 300 mg/kg compared to the untreated negative control group, which displayed a continuous decrease in RBC levels. Additionally, the increase in RBC observed in the positive control group treated with 10 mg/kg of Artesunate was comparable to the rise achieved with the extract at 300 mg/kg. (Table 4).

Table 4: Influence of *vernonia amygdalina* leaf extract on red blood cell count in mice with established malaria infection (Curative Test)

Extract/drug	Dose	RBC count 5th day	RBC count 6th day	RBC count 7th day
Control	--------	10.05±0.07	10.05±0.07	10.05±0.07
Normal saline	2ml/kg	4.25 ± 0.02	3.77 ± 0.16	3.63 ± 0.21
V. amygdalina	150mg/kg	7.17± 0.17*	7.21± 0.13*	8.14± 0.14*
V. amygdalina	300mg/kg	7.06± 0.09*	8.08± 0.06*	8.57± 0.19*
Artesunate	10mg/kg	6.92± 0.07*	8.44± 0.13*	8.70± 0.18*

*$p<0.001$ indicates a significant increase compared to the negative control group; N = 5. Data are presented as Mean ± SEM

Discussion

The phytochemical analysis of the ethanol leaf extract of *Vernonia amygdalina* revealed the presence of several compounds, including tannins, alkaloids, saponins, cardiac glycosides, flavonoids, steroids, and phenols. Total flavonoid and phenolic contents of this extract were 4.62±0.50 and 46.9±0.96 respectively indicating moderate to high phytochemical components. This result is in conformity with earlier findings that ethanol extract of *Vernonia amygdalina* contained secondary metabolites [29]. High phenolic components of the extract as found in this study may be responsible for its antioxidant effects as earlier reported that phenolics possess antioxidant properties which helps prevent oxidative damage of cells by neutralizing free radicals through its scavenging activity [30].

Acute oral toxicity study on this extract reveals its nontoxic nature in all the doses administered, except in 3200mg/kg/bwt in which they showed restlessness and labored breathing but no death. Thus the acute toxicology study has confirmed that it can be classified as safe to be consumed. This result aligns with earlier studies indicating oral administration of plant extract 100 times safer than intraperitoneal administration [31], but contradicts the findings that recorded an LD_{50} of 1950 mg/kg/body weight of *V. amygdalina* extract with signs of toxicity at a dose of 3000mg/kg [32]. Variations in the LD50 of *Vernonia amygdalina* extract may be attributed to factors such as the extraction method, the dosage administered, or other influencing variables.

Antioxidant enzymes serve as the primary defense against cellular damage, helping to mitigate harmful effects [33]. Malondialdehyde (MDA) is commonly used in biochemical assays to assess the degree of oxidative damage in cells. The results indicated a significant decrease ($p<0.001$) in MDA levels in groups treated with *Vernonia amygdalina* extract at doses of 150 mg/kg and 300 mg/kg compared to negative control group, which received only normal saline (see Figure 3). This suggests that extract helps reduce lipid peroxidation by enhancing antioxidant defenses and minimizing damage to cell membranes. This is in agreement with the findings which recorded decrease in the MDA level of *V. amygdalina* treated groups at the doses of 10mg/kg and 50mg/kg [34]. The slight reduction in MDA levels observed in this study at doses of 150 mg/kg and 300 mg/kg could be attributed to the effects of the extract.

The ethanol leaf extract of *Vernonia amygdalina* administered at doses of 150 mg/kg and 300 mg/kg led to a significant

increase in glutathione activity compared to the negative control group, which exhibited a statistically significant decrease in GSH activity ($p<0.001$). The observed rise in glutathione activity among the extract-treated groups suggests improved cellular resilience, enhanced detoxification capabilities, and better protection against oxidative stress and damage. These results align with previous findings that reported a significant increase in reduced glutathione activity in mice treated with *Vernonia amygdalina* extract at doses of 200 mg/kg, 400 mg/kg, and 600 mg/kg body weight [35].

Catalase is a vital intracellular antioxidant enzyme that plays a key role in safeguarding cells from oxidative damage by breaking down hydrogen peroxide into water and oxygen. The results of this study revealed a significant decrease ($p<0.001$) in serum catalase activity in mice treated with 150 mg/kg and 300 mg/kg of *Vernonia amygdalina* extract compared to uninfected group (Baseline), as illustrated in Figure 3. Decrease in serum catalase in the extract treated groups suggests reduced antioxidant capacity, higher oxidative stress and susceptibility to cellular damage from oxidative stress. This is in line with the findings that reported significant decrease in serum catalase activity of *Vernonia amygdalina* extract in malaria treated mice [36].

The *in-vitro* antioxidant capacity of *Vernonia amygdalina* leaf extract was assessed using the DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) assay, a widely recognized method for evaluating the antioxidant activity of plants. This assay revealed a fading of the violet color in the presence of substances capable of donating hydrogen, which reflects their antioxidant potential. The findings indicated that the ethanol leaf extract of *V. amygdalina* moderately scavenged DPPH free radicals, demonstrating a scavenging power of 68% at the highest concentration tested (500µg/ml). However, this activity was not as effective as that of the standard antioxidant, ascorbic acid, which achieved a 92% scavenging rate at the same concentration (Figure 1).

The activity of extract in decomposing hydrogen peroxide exhibited a concentration-dependent pattern. At the highest concentration tested, 500 µg/ml, *Vernonia amygdalina* leaf extract scavenged 50% of hydrogen peroxide; however, this was not as effective as ascorbic acid, which demonstrated a 91% scavenging rate at the same concentration (Figure 2). This result contrasts with previous findings that reported a hydrogen peroxide scavenging activity of 85.8% for *V. amygdalina* extract [37]. The discrepancies could be attributed to variations in the extraction method, the concentrations used, or other influencing factors.

Oral administration of *Vernonia amygdalina* extract for seven days in a curative approach to established malaria infection led to an increase in the packed cell volume (PCV) of mice treated with doses of 150 mg/kg and 300 mg/kg. This increase in PCV was particularly noticeable at the 300 mg/kg dose, showing higher levels compared to both the 150 mg/kg dose group and the Artesunaaate-treated group (10 mg/kg). In contrast, the negative control group experienced a marked and significant decline in PCV after four days of treatment (Figure 5). These findings differ from previous research [38], which reported no significant change in red blood cell count, PCV, or total white blood cell count with *V. amygdalina* extract compared to negative control. Additionally, the *V. amygdalina* leaf extract significantly ($p<0.001$) increased the red blood cell count at both 150 mg/kg and 300 mg/kg doses, whereas the negative control group showed a consistent decrease in RBC. Also, increase in RBC observed in positive control group treated with 10mg/kg of artesunate was comparable with increase caused by the extract at 300mg/kg. The rise in red blood cell (RBC) count in the groups treated with the extract from day 5 to day 7 aligns with reports that *Vernonia amygdalina* extract can help prevent drastic reductions in RBC levels [39]. This resulted in significant increases in both RBC count and packed cell volume, suggesting that the extract has erythropoietic effects. The boost in RBC count, particularly at the higher dose of 300 mg/kg, was comparable to the response seen in the artesunate-treated group. In contrast, the negative control group showed a notable decrease in RBC count compared to the treated groups.

Impact of *V. amygdalina* leaf extract in early malaria infection was ascertained using suppressive test, a reliable and standard method for determination of percentage parasitaemia suppression. Two doses of the extract used in this study (150mg/kg and 300mg/kg) suppressed the parasite growth in the early malaria infection by 63.7% and 77% respectively which was comparable to 81.7% suppression of parasite by artesunate. (Table 2) This correlates with the findings that leaf extract of *V. amygdalina* produced 67% and 53.5% suppression of parasitaemia in four-day suppressive test respectively at doses of 500mg/kg and 250mg/kg[40] respectively.

Curative effect of *V. amygdalina* extract in established malaria infection showed significant ($p<0.001$) decrease in parasitaemia of extract-treated groups when compared to negative control group. Artesunate, an ACTs based standard antimalarial drugs inhibited parasite growth by 78.4% at 10mg/kg while *V. amygdalina* extract resulted in parasitaemia inhibition by 66.2% and 75.8% at doses of 150mg/kg and 300mg/kg respectively (Table 3). Effect of the extract in parasitaemia inhibition at 300mg/kg was comparable to that of artesunate antimalarial drug used at 10mg/kg. Significant increase observed in parasite count of negative control group both on 5^{th} day (58.36 ± 0.43) and 7^{th} day (64.52 ± 0.74) is due to malaria infection which is steadily progressing. Parasitaemia suppression of *V. amygdalina* extract at doses of 150 mg/kg and 300mg/kg at 66.2% and 75.8% respectively is considered good, this is in accordance with the findings[41] that *in-vivo* anti-malarial activity of an extract is considered as very good, good and moderate if suppression of parasitaemia is $\geq$ 50% at 100 mg/kg, 250 mg/kg and 500 mg/kg body weight/day, respectively. (Table 2 and Table 3).

In established malaria infection, mice treated with *Vernonia amygdalina* leaf extract at doses of 150 mg/kg and 300 mg/kg survived up to 25 and 27 days, respectively. In comparison, the group treated with Artesunaaate survived for 28 days or longer, whereas the negative control group survived only 13 days. These findings align with previous research[42] indicating extended mean survival times in groups treated with the highest dose of plant extract and standard drug relative to the negative control. Thus, *Vernonia amygdalina* leaf extract effectively inhibited parasite growth, helped maintain packed cell volume in treated mice, and prolonged their survival time.

Conclusion

The data from this study indicate that *Vernonia amygdalina* leaf extract acts as a moderate scavenger of free radicals and demonstrated potent antimalarial activity which is comparable to activity exhibited by artesunate, an ACT-based synthetic antimalarial drug used and can be harnessed for the production of antimalarial pharmaceuticals. However, the extract can be further accessed for more toxicity test due to low-level of catalase enzyme and for use in treatment of

anemia.

Ethical considerations
All ethical considerations guiding the use of animals for research were duly followed.

Authors Contribution
We all contributed actively to achieve success in this work both in and out of the laboratory.

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Competing Interest
We conducted this research and to the best of our knowledge, there's no competing interest.

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