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Antimalarial Activity of Crude Extract and Fractions of *Phyllanthus amarus* in *Plasmodium berghei*-Infected Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author AUB performed the experiments, managed the analyses of the study, literature searches and wrote the draft of the manuscript. Authors MA and KAY supervised the work and managed the proof reading. Author OEO designed and supervised the work and managed the proof reading. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study evaluated the antimalarial activity of the crude extract and fractions of *Phyllanthus amarus* in *Plasmodium berghei*-infected mice.
Place and Duration of Study: Department of Biochemistry, Federal University of Technology, Minna, Niger State, Nigeria, between February 2016 and August 2016.
Methodology: Mice infected with *Plasmodium berghei* were administered orally with the crude extract of *Phyllanthus amarus* whole plant 72 hours post infection at doses ranging from 100-500 mg/kg/day, for five consecutive days. Chloroquine (5 mg/kg/day).and artesunate (50 mg/kg/day) were used as controls, while distilled water was administered to the negative control groups. N-

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hexane, chloroform, ethyl acetate and aqueous fractions, obtained from crude aqueous methanolic extract, were also evaluated for their inhibitory effect against *P. berghei* at doses ranging from 50-200 mg/kg/day. Level of parasitaemia, survival time, variations in the values of body weight and % PCV were monitored throughout the study period.

Results: Crude extract of *Phyllanthus amarus* whole plant showed significant ($P < 0.05$) antiplasmodial activity in dose dependent pattern with 76.74% inhibition of parasite growth. Aqueous fraction at a dose of 200 mg/kg demonstrated significant antiplasmodial activity with %inhibition of parasite growth of 56.40. The variations in the values of weight and %PCV before and after treatment were not significant in both the crude and aqueous fraction. Significant inhibition of parasite growth by the crude extract and aqueous fraction resulted in longer mouse survival relative to the control, as confirmed in the mean survival time of the mice (27.67±1.45, 22.67±0.67, 29.33±0.67 and 6.67±0.88 days) for the crude extract (500mg/kg), aqueous fraction (200 mg/kg), chloroquine and negative control groups respectively. Phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, phenol, tannins, steroids, terpenoids and saponins.

Conclusion: The results indicate that the whole plant extract and fractions of *Phyllanthus amarus* have antimalarial property which can serve as a novel source for the development of new and affordable antimalarial agent.

Keywords: Phyllanthus amarus; Plasmodium berghei; curative; antimalarial; malaria.

1. INTRODUCTION

After many years of man's struggle with malaria, the disease is still an important health problem in the tropics. The disease is prevalent in only tropical and sub-tropical countries of the world. This is due to the fact that the malaria parasite requires two different hosts (the human and the mosquito) and tropical climate coupled with unhygienic environment of the poor nations favour mosquito breeding. According to the Malaria Report of 2016, 212 million cases of malaria occurred in 2015 with over 429 000 deaths particularly in sub-Saharan Africa, where an estimated 90% of all malaria deaths occur [1].

Malaria is a life-threatening parasitic disease caused by the intracellular, protozoan parasites of *Plasmodium* species that are transmitted to people through the bites of infected female *Anopheles* mosquitoes. Malaria in humans is caused by five *Plasmodium* species, namely, *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi*. Of these, *P. falciparum* is the causative agent of severe malaria and the major cause of malaria-related mortality [2,3].

In Nigeria, malaria is endemic throughout the country. Nigeria suffers the world's greatest malaria burden, with approximately 10 million cases and 300,000 deaths reported annually, while 97% of the total population (approximately 173 million) is at risk of infection [4,5]. Moreover, malaria accounts for 60% of outpatient visits to health facilities, 30% childhood death and 11 % maternal mortality [4-7]. The financial loss due to malaria annually is estimated to be about 132 billion naira in form of treatment cost, prevention and loss of man-hours [5,8].

In the absence of a viable malaria vaccine, the methods of controlling malaria which include chemotherapy and vector control are beset with problems of resistance of *Plasmodium* parasite to currently available antimalarial drugs and malaria vector to a wide range of insecticides. Therefore, there is an urgent need for the discovery and development of novel and affordable antimalarial drugs and of new treatments.

Traditional medicine for malaria treatment are already in popular use in developing countries including Nigeria and many have been shown to have antimalarial activities in experimental studies. Plant products, artemisinin and quinine derivatives, are the source of the two core current antimalarial drugs [9]. Plant materials are endowed with natural products with interesting chemical structures and promising biological activities.

Phyllanthus amarus is widely used as a medicinal plant and is considered to be a good tonic, diuretic and febrifuge [10]. It is used in the Indian Ayurvedic system of medicine in problems of stomach, genitourinary system, liver, kidney and spleen [10]. There has been considerable research into the medicinal properties of the plant, much of it supporting the traditional uses.

The crude extract of *Phyllanthus amarus* whole plant has been reported to possess antimalarial activity [8,11,12]. In our present study we further evaluated the antimalarial activity of the whole plant crude extract as well its fractions. All the fractions were tested for antimalarial activity and their effects on PCV and body weight on *Plasmodium berghei* infected mice were determined.

2. MATERIALS AND METHODS

2.1 Plant Samples

Fresh whole plant of *Phyllanthus amarus* was collected in July, 2015 from Minna, Niger State, Nigeria. The plant was identified by a taxonomist at the Botany Department of University of Nigeria Nsukka, Enugu State. A voucher number of UNH 95 was assigned and deposited at the herbarium of Botany Department of University of Nigeria, Nsukka, and Enugu State, Nigeria.

2.2 Parasites

Plasmodium berghei (NK65) was obtained from the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. It was maintained in mice by serial blood passage in mice.

2.3 Animals

Adult Swiss albino mice, male and female, weighing between 22-28g were obtained from National Veterinary Research Institute (NVRI), Vom, Plateau State of Nigeria and were acclimatized in the experimental animal house for two weeks. Animals were fed with standard mice/rat pellet diet (Vital Feeds, Nigeria) and water *ad libitum*. The experiment was conducted in strict compliance with the internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care (CCAC) guidelines on animal use protocol review [13].

2.4 Preparation of Crude Extract

Phyllanthus amarus (whole plant) were collected, washed, air dried at room temperature to a constant weight and pulverised using a blender. Fifty grams of the powdered sample was extracted under reflux in 400ml of 70% methanol for 2 h with Medline extraction mantle according to the method of [14]. The extracts were filtered hot through muslin cloth, and then concentrated to dryness under reduced pressure using a rotary evaporator. The extracts were transferred into sterile universal tubes, and kept in the refrigerator until required for use.

2.5 Fractionation of the Crude Extracts

The aqueous methanolic extract of *Phyllanthus amarus* was further fractionated by solvent fractionation technique. Twenty four grams (24 g) of the crude extract of *P. amarus* was dissolved in 100ml of water in a beaker to give the aqueous layer. The mixture of the extract was poured into a one litre separatory funnel and then successively and exhaustively partitioned by fractionation using n-hexane, chloroform and ethyl acetate. Four fractions (n-hexane, chloroform, ethyl acetate and residual aqueous) were obtained. Organic fractions were concentrated *in vacuo* by the evaporation of the various solvent soluble extracts under reduced pressure at 35°C using rotary evaporator, while aqueous fractions were concentrated by freeze drying.

2.6 Parasite Inoculation

Blood samples were collected from the tail veins of previously *P. berghei* infected donor mouse having a parasite load of 20–30% to infect the mice. Subsequently, the blood was diluted in normal saline (0.9%) so that the final suspension contained 10^7 infected red blood cells in each 0.2 ml of the preparation. Accordingly, healthy mice were inoculated with 10^7 *P. berghei* parasitized RBCs via the intraperitoneal route [15,16].

2.7 Antimalarial Activity of the Crude Extract of *Phyllanthus amarus*

The curative potential of plant extracts was evaluated by employing the method of [17]. On the 1st day (day 0), 21 albino mice were infected with a standard inoculum of *P. berghei* infected red blood cells intraperitonealy. Seventy-two hours later (day 3), following confirmation of parasitaemia, these infected mice were randomly assigned into 7 groups of 3 mice each and treated orally with the extract at doses of 100 (A), 300 (B) and 500 (C) mg/kg/day. The positive control groups (D& E) were treated with standard drugs, 5 mg/kg/day chloroquine and 50 mg/kg/day artesunate, respectively and 0.2 ml distilled water was administered to group F (negative control group). Group G were not infected but treated with 500 mg/kg body weight of the extract. All the treatments lasted for 5

days. Giemsa stained (10%) thin blood film was examined microscopically. Average percentage parasitaemia and inhibition of parasite growth were calculated according to the following formulae [18]:

% parasitaemia =

$$\frac{\text{Number of parasitized erythrocytes}}{\text{Total number of erythrocytes}} \times 100$$

$$\% \text{ inhibition} = \frac{A-B}{A} \times 100$$

Where:

A = Parasitaemia in negative control
B = Parasitaemia in test group

2.8 Antimalarial Activity of Fractions of *Phyllanthus amarus* Extract

A total of 45 mice were infected with *Plasmodium berghei* on the 1st day (day 0). Seventy-two hours later (day 3), following confirmation of parasitaemia, these infected mice were randomly assigned into 15 groups of 3 mice each and treated orally with the various fractions each at doses of 50, 100 and 200 mg/kg/day for five days. The positive control group was treated with chloroquine, 5 mg/kg/day and 0.2 ml distilled water was administered to negative control group while another group received the crude extract (500 mg/kg/day). Giemsa stained (10%) thin blood film was examined microscopically. Average percentage parasitaemia and inhibition of parasite growth were calculated using the formula described above.

2.9 Weight Determination

The body weight of each mouse in all the groups was taken before infection (day 0) and after treatment (day 8) using a sensitive weighing balance.

2.10 Determination of Packed Cell Volume

Blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes. The capillary tubes were filled with blood up to ¾th of their volume and sealed. The tubes were sealed by crystal seal and placed in a microhematocrit centrifuge with sealed ends outwards and centrifuged for 5 min at 11,000 rpm. The PCV was measured before inoculating the parasite (day0) and after treatment (day8)

2.11 Determination of Mean Survival Time

Mortality of the experimental mice was closely observed daily and the number of days from the time of infection with *P. berghei* up to the incidence of death was recorded for every mouse in both the treated and control groups during the experimental period.

Mean Survival Time (MST) of all mice were determined using the formula:

MST =
Sum of survival time of all mice in a group (days)
Total number of mice in that group

2.12 Phytochemical Screening

Phytochemical Screening was performed on the crude extracts using standard procedures to identify the secondary metabolites as described by [19,20].

2.13 Statistical Analysis

Results of the study were expressed as mean ± SEM. Data were analysed using Windows SPSS Version 19.0. One-way analysis of variance (ANOVA) coupled to Duncan (post-hoc test) was used to determine statistical significance for comparison of parasitaemia, % inhibition, body weight, PCV, survival time, haematological and biochemical parameters among groups. For all the data obtained, the result was considered significant at 95% confidence level and P-value < 0.05.

3. RESULTS

3.1 Antiplasmodial Activity of Crude Extract of *Phyllanthus amarus* Whole Plant

In mice infected with *Plasmodium berghei,* the aqueous methanolic extract of *Phyllanthus amarus* whole plant demonstrated significant ($P < 0.05$) and dose dependent antiplasmodial activity after five days of treatment (Fig. 1). The level of parasitaemia at 100, 300, and 500mg/kg was reduced by 58.90, 65.91, 76.74% respectively as against 84.18 and 86.30% for the standard drugs chloroquine and artesunate respectively (Table 1).

There was no significant (P > 0.05) change in body weight and %PCV in all the test groups before infection and after 5 days of treatment, however those mice in the infected but not treated group showed significant (P < 0.05) weight loss and PCV reduction (Fig. 2 and 3). Survival dates ((Table 1)) were significantly prolonged by all dose levels as compared with untreated control group. Increase in body weight and PCV was observed in the group that was not infected but treated (NIT) although the increase was not significant (P > 0.05) (Fig. 2 and 3).

3.2 Antiplasmodial Activity of Fractions of *Phyllanthus amarus* Extract

The results for the antiplasmodial activity of four fractions obtained from solvent - solvent partitioning of *Phyllanthus amarus* are presented

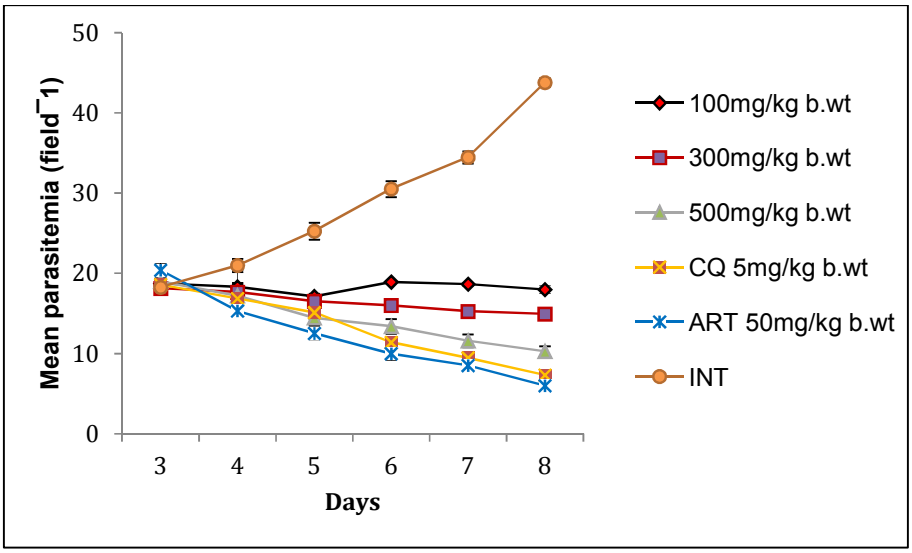

Fig. 1. Antiplasmodial activity of crude extract of *Phyllanthus amarus*
Data are mean ± SEM, n = 3, CQ = chloroquine, ART = artesunate, INT = infected but not treated

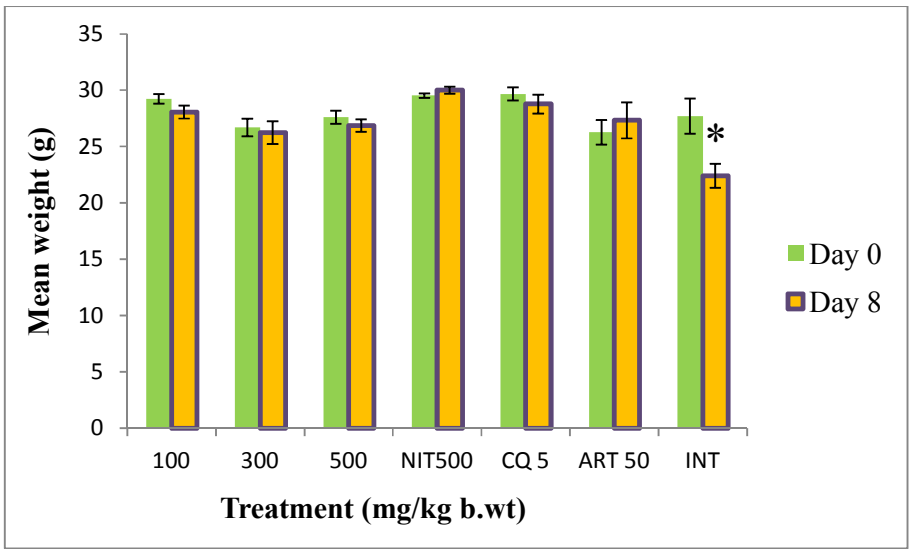

Fig. 2. The effect of crude extract of *Phyllanthus amarus* on body weight of *P. berghei* infected mice
*Data are mean ± SEM; n = 3, CQ = chloroquine, ART = artesunate, NIT = not infected but treated, INT = infected but not treated, * = significant difference between the weight before and after treatment (P < 0.05)*

Table 1. Effect of aqueous methanolic extracts of *Phyllanthus amarus* on the % inhibition of parasite growth and survival time of *P. berghei* infected mice

Dose (mg/kg)	Parasitaemia (Day 8)	% Inhibition	Mean survival time (Days)
100	18.00±0.47	58.90	21.67±3.26
300	14.93±0.29	65.91	26.00±2.31
500	10.00±0.66	77.17	28.67±0.88
500 (NIT)	-	-	30.00±0.00
5 (CQ)	7.93±0.44	81.90	28.00±1.16
ART 50	6.00±0.50	86.30	29.33±0.67
INT	43.80±0.53	0	10.33±0.33

Values are expressed as mean ± SEM, n = 3, Art = Artesunate, CQ = Chloroquine diphosphate, INT = Infected but not treated (untreated control), NIT = not infected but treated, - not done

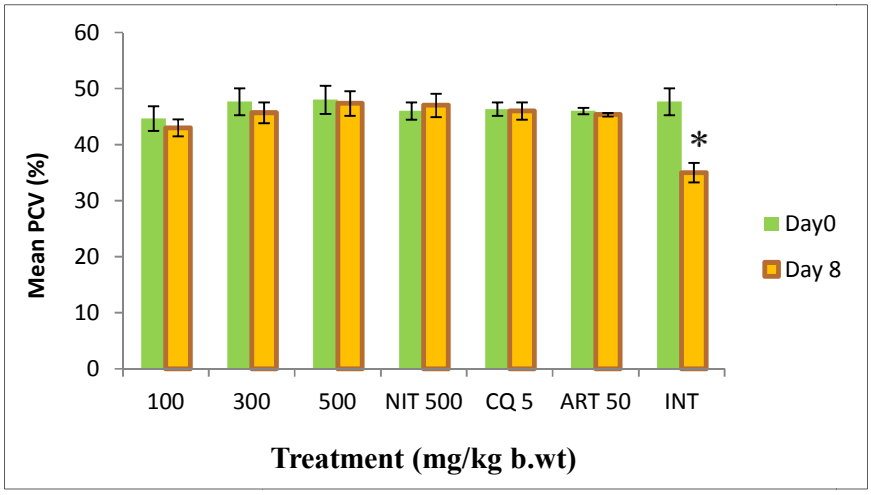

Fig. 3. The effect of crude extract of *Phyllanthus amarus* on PCV of *P. berghei* infected mice

*Data are mean ± SEM; n = 3, CQ = chloroquine, ART = artesunate, NIT = not infected but treated, INT = infected but not treated, * = significant difference between the %PCV before and after treatment (P < 0.05).*

in Table 2. Among the fractions, the aqueous fraction at the dose of 200 mg/kg body weight demonstrated the best antiplasmodial activity and reduced the level of parasitaemia by 56.40% when compared to the untreated control after 5 days of treatment. However, the inhibitory effect was significantly ($P < 0.05$) less when compared to crude extract (79.24%) and chloroquine. Variations in weight was not significant ($P > 0.05$) in all the groups except in the groups treated with hexane fraction at the dose of 200 mg/kg body weight, chloroform fraction at the dose of 200 mg/kg body weight, ethyl acetate fraction at 100 and 200 mg/kg body weight dose levels and untreated control (Fig. 4). Significant ($P < 0.05$) reduction in percentage packed cell volume was observed in all the groups except the groups treated with aqueous fraction at the dose of 200mg/kg body weight, crude extract and chloroquine (Fig. 5). Mean survival times (Table 2) of fraction-treated groups were significantly ($P < 0.05$) longer when compared to untreated control but shorter when compared with the standard drug (chloroquine).

3.3 Phytochemical Screening

Phytochemical screening of the crude extract of *Phyllanthus amarus* whole plant revealed the presence of alkaloids, flavonoids, tannins, phenols, steroids, terpenoids and saponins.

4. DISCUSSION

It has been described that plant contain active compounds that have great potential for medicinal use, and both traditional healers and pharmaceutical drug companies make use of these plants [21, 22, 23].

The rodent parasite, *P. berghei,* has been widely employed in the discovery and development of several conventional antimalarial drugs validated by quinine, halofantrine, chloroquine, mefloquine

Table 2. Effect of aqueous methanolic extracts of *Phyllanthus amarus* on the % inhibition of parasite growth and survival time of *P. berghei* infected mice

Dose (mg/kg)	Parasitaemia (day 3)	Parasitaemia (Day 5)	Parasitaemia (Day 8)	%Inhibition	Mean Survival time (days)
H50	14.67±.083	14.42±0.47	21.50±0.66	10.71	13.33±0.67
H100	14.25±0.58	15.58±0.46	21.08±1.22	12.46	13.33±1.20
H200	14.75±0.14	14.83±0.56	20.67±0.36	14.16	15.33±1.45
C50	14.50±0.38	14.67±0.51	16.33±0.22	32.18	13.67±1.45
C100	14.58±0.55	15.75±0.52	14.58±0.18	39.45	15.67±1.76
C200	14.67±0.22	15.00±0.14	14.50±1.30	39.78	13.67±0.67
E50	13.92±0.22	13.17±0.33	16.67±0.42	30.77	16.33±1.45
E100	14.08±0.17	12.75±0.52	15.83±0.99	34.26	14.67±0.33
E200	14.33±0.58	13.42±0.46	15.00±0.52	37.70	13.33±1.45
A50	14.83±0.36	13.17±0.38	13.00±1.00	46.01	20.67±0.88
A100	14.67±0.39	13.20±0.08	12.00±0.67	50.17	20.67±1.76
A200	14.08±0.17	12.75±0.25	10.50±0.25	56.40	22.67±0.67
Cr500	14.17±0.28	10.25±0.42	5.00±0.80	79.24	27.67±1.45
CQ 5	14.00±0.38	10.08±0.33	4.25±0.52	82.35	29.33±0.67
INT	14.08±0.30	16.33±0.66	24.08±0.42	0	6.67±0.88

Data are mean ± SEM; n = 3, H = hexane fraction, C = chloroform fraction, E = ethyl acetate fraction, A = aqueous fraction, Cr = crude extract, CQ = chloroquine, INT = infected but not treated (untreated control).

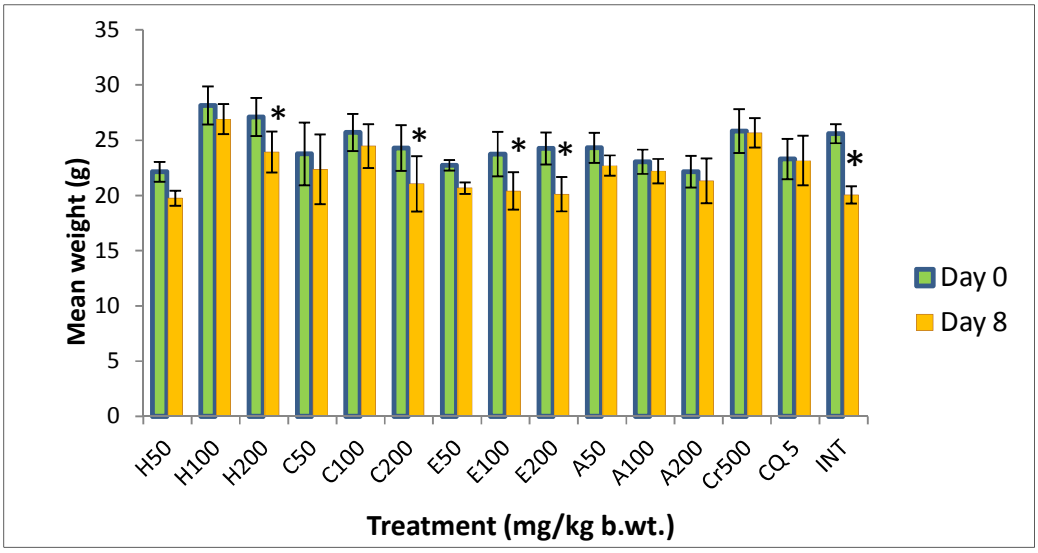

Fig. 4. Effect of fractions of *Phyllanthus amarus* on body weight of mice infected with *Plasmodium berghei*

*Data are mean ± SEM; n = 3, H = hexane fraction, C = chloroform fraction, E = ethyl acetate fraction, A = aqueous fraction, Cr = crude extract, CQ = chloroquine, INT = infected but not treated, * = significant difference between the weight before and after treatment (P < 0.05)*

and more recently artemisinin derivatives [22,24, 25].

The *in vivo* antiplasmodial activities of the aqueous methanolic extracts and fractions of *Phyllanthus amarus* whole plant were investigated by evaluating the antimalarial activity during established infection using rodent models. Determination of percentage inhibition of parasite growth is regarded as the most dependable parameter in antimalarial drug discovery [26,27]. A mean parasitemia level ≤ 90% to that of mock-treated control animals usually indicates that the test compound is active in standard screening studies [28,29].

In the 5-day curative test, treatment of infected mice with crude extract of *Phyllanthus amarus*

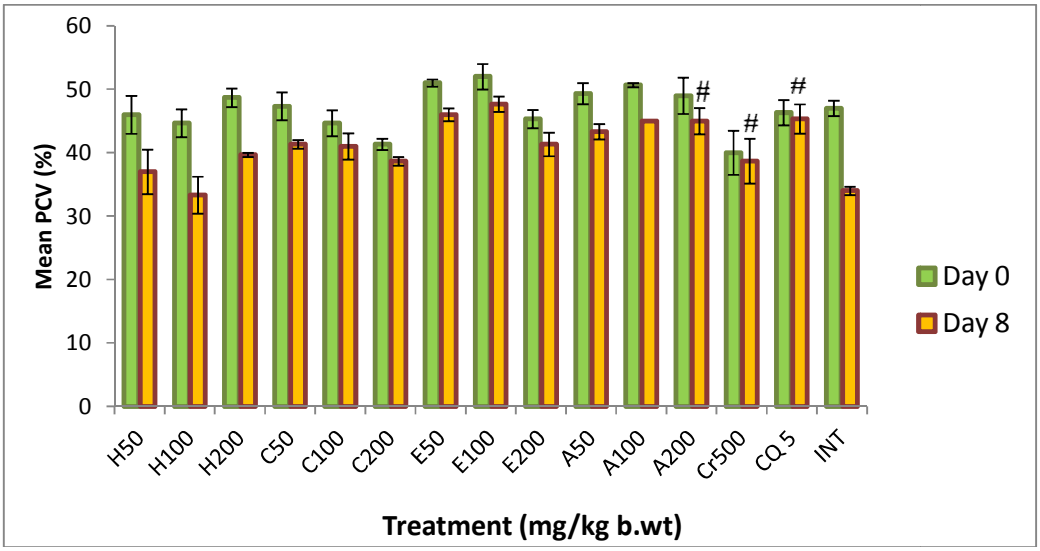

Fig. 5. The effect of fractions of *Phyllanthus amarus* on PCV of *P. berghei* infected mice
Data are mean ± SEM; n = 3, H = hexane fraction, C = chloroform fraction, E = ethyl acetate fraction, A = aqueous fraction, Cr = crude extract, CQ = chloroquine, INT = infected but not treated, # = no significant difference between the %PCV before and after treatment (P > 0.05)

(whole plant) significantly inhibited parasite growth in a dose-dependent manner signifying that the plant is endowed with antimalarial potential. [8,11,12] reported the antimalarial activity of the aqueous extract of *Phyllanthus amarus* leaf and whole plant.

Anaemia and body weight loss are general characteristics of *P. berghei* infected mice [27]. Plants with antimalarial activity are expected to prevent body weight loss in infected mice due to rising parasitaemia that results in loss of appetite. *Plasmodium berghei* infected mice suffer from anaemia because of erythrocyte destruction as the presence of many abnormal erythrocytes stimulates the spleen to produce many phagocytes [22,30]. The crude extract of *Phyllanthus amarus* significantly prevented reduction in PCV as well as body weight loss associated with rising parasitemia. The ability of this extract to alleviate these common pathological features of *P. berghei* infection suggests its antimalarial efficacy.

In evaluating the anti-malarial activity of plant extracts, the mean survival time is essential since it is related to parasite growth inhibition [18]. The mean survival time of the group treated with crude extract of *Phyllanthus amarus* was significantly ($p<0.05$) prolonged when compared with the negative control signifying that it inhibited the *growth of P. berghei* and ameliorated the pathological effects of the parasite on the mice. Though, the crude extracts and the standard drug did not cure the infection. Hence, recrudescence of *P. berghei* parasites after apparent cure could account for this observation.

Some secondary metabolites of plants such as alkaloids, flavonoids, phenols and terpenoids have been reported to possess antiplasmodial activity [31-34]. These compounds were found to be present in the crude extract and may account for the observed antimalarial activity of the extract.

The reduction in body weight gain and PCV is a simple and sensitive tool in toxicological study after exposure to toxic substance [35]. The crude extract administered to non-infected normal mice at 500 mg/kg body weight per day for five days did not result in body weight loss and PCV reduction of the test animals. Instead an increase in body weight and PCV were recorded. The 30 days observation period elapsed without recording any death in all the experimental mice. This suggests that the crude extract is safe for oral administration at the dose tested.

Fractions of *Phyllanthus amarus* crude extract showed varying degrees of inhibition of malaria parasite growth. The aqueous fraction was found to be most potent. This was evident from the chemoinhibition obtained during the five day curative test and suggests that the active

ingredients are resident in this fraction. This fraction also prevented reduction in body weight and PCV significantly at the maximum dose administered. This implies that the aqueous fraction alleviated the common pathological effects of malaria parasite. The mean survival time of mice treated with the aqueous fraction was significantly longer than the mice treated with the other fractions but shorter than the crude extract treated mice. The longest survival time of mice as a result of the administration of the highest dose of aqueous fraction could be connected to the presence of active phytochemicals in adequate concentration in that dose. However, the percentage inhibition of parasite growth by the crude extract was significantly higher than the aqueous fraction. This suggests that fractionation of the crude extract must have resulted in a partial loss of activity. This may also imply that the antiplasmodial activity of the crude extract could be due to synergistic action of the bioactive components. Our findings are supported by similar observation reported by other investigators [14, 36]. Though, *in vivo* antiplasmodial activity of plant extracts are classified as moderate, good and very good if an extract displays parasite inhibition ≥ 50% at respective doses of 500, 250 and 100mg/kg body weight per day [33, 37]. The aqueous fraction can be considered as having good antiplasmodial activity since it demonstrated > 50% growth inhibition when administered at a dose of 200 mg/kg body weight per day for five days. Hence, further purification and isolation of the bioactive compound(s) could lead to a better antimalarial activity.

5. CONCLUSION

Our results demonstrated that the crude extract and the aqueous fraction of *Phyllanthus amarus* whole plant possess antimalarial activity. Further studies on the most active fraction can lead to isolation of novel antimalarial agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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