Pakistan Journal of Biological Sciences 16 (11): 510-516, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.510.516

Evaluation of Haematological Changes in *Plasmodium-berghei*-infected Mice Administered with Aqueous Extract of *Phyllantus amarus*

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Abstract: This study was designed to evaluate the changes in some hematological parameters of *P-berghei*-infected mice treated with aqueous extract of *Phyllantus amarus*, a plant that is used traditionally to treat malaria patients in some Nigerian communities. The aqueous extract of the leaves at 200, 400 and 600 mg kg^{-1} body weight/day dose levels were used to treat the test groups immediately after infection for the suppressive test and 72 hours post infection for the curative test while a standard antimalarial drug, Artesunate, at a dose of 50 mg kg^{-1} body weight was administered on the positive control group. The negative control group was left untreated. The level of parasitemia, variation in weight, Percentage Packed Cell Volume (% PCV), erythrocytes (RBC) and leukocytes (WBC) counts in the different groups were monitored throughout the period of study. The crude extract was screened for its phytochemical composition. The crude extract at 200, 400 and 600 mg kg^{-1} body weight/day suppressed parasitemia by 54.67, 61.25 and 61.24% after treating for four days in the suppressive test as against 72.32% for the standard drug while the level of parasitemia was reduced by 64.35, 66.71 and 67.13%, respectively after treating for five days in the curative test as against 71.87% for the standard drug. The variations in the values of Percentage Packed Cell Volume (%PCV), weight, leukocyte and erythrocyte counts for treated groups before and after treatment was not significant ($p<0.05$). Alkaloids, flavonoids, tannins, glycosides, saponin, carbohydrate and phenols were found to be present in the crude extract. The findings of this study show that the use of *Phyllantus amarus* as antimalaria regimen by local medical practitioners does not adversely affect the weight and the haematological parameters determined.

Key words: *Phyllantus amarus*, *Plasmodium berghei*, suppressive, curative, leukocyte, erythrocyte

INTRODUCTION

Malaria affects millions around the world but a reliable estimate of those infected and at risk from the disease has been difficult to come by. In many parts of Africa, children suffer more than one clinical bout of malaria each year, resulting in death or life-long health problems from delayed treatment. Most often public health officials and aid agencies do not know which regions are most affected (Avasthi, 2005).

Despite decades of research, malaria infection remains a major global health problem with high mortality and morbidity than any other infectious disease (World Health Organization, 2011). Efforts to disrupt the life cycle of the parasite by controlling the vector have had only limited success while the usefulness of anti-malarial drugs is hampered by their lack of availability to those most in need and to the rapid evolution of drug resistant parasites. An effective vaccine remains the most promising approach to controlling the disease (Good, 2009) but is still not available. Research on malaria vaccines is complicated by the complexity of the parasite life cycle. To complicate matters, reported mechanisms of protection against the liver stage of the parasite differ not only between human and rodent malaria species but also between different rodent malaria species.

Malaria occurs in over 90 countries worldwide. According to figures provided by the World Health Organization 36% of the global population live in areas where there is risk of malaria transmission, 7% reside in areas where malaria has never been under meaningful control and 29% live in areas where malaria was once transmitted at low levels or not at all, but where significant transmission has been re-established (World Health Organization, 2011).

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Geographically, malaria overlaps with other infectious maladies including HIV which often complicates the illness as well as treatment options. The resistance of Plasmodium spp. to drugs-such as chloroquine-has become a serious problem in areas of endemic malaria and in malaria-free areas with occasional imported cases. One possible approach to the identification of new antimalarial drug candidates is to search for compounds that cure or prevent malaria in plants empirically used to treat malaria. There is also a need to generate reliable scientific data to determine whether the plants currently used to treat malaria are actually effective and have no adverse effects on the system. In the long term, this should help to prevent deaths due to ignorance and the misuse of plants for self-medication in the absence of advice from a qualified medical professional (Milijaona *et al*., 2003).

According to latest estimates from the World Health Organization (WHO), in 2009, there were 225 million cases of malaria and an estimated 781,000 deaths worldwide-most deaths occurring among children living in the WHO African Region (mainly sub-Saharan Africa) (World Health Organization, 2011).

In Nigeria, malaria is endemic throughout the country. World Health Organization (WHO) estimated malaria mortality rate for children under five in Nigeria at 729 per 100, 000. The Ministry of Health reported in April 2004 that malaria is responsible for one out of ten deaths in pregnant women and has caused the Federal Government of Nigeria over one billion Naira annually in treating malaria (Odugbemi *et al*., 2007).

Under the circumstances, scientists in Nigeria and other parts of the world are exploring many approaches, targeting different stages of the parasite life cycle, to find agents that will prevent, cure, or eliminate malaria. These approaches include new drug discovery, old drug rehabilitation and drugs borrowed from other fields for use against malaria. The need for new drugs has led to research on plants with medicinal value.

Medicinal plant, according to Sofowora (1993), is one which one or more of its organs contain substances that can be used for therapeutic purposes. It may be in form of vegetable drug which may either be organized material which possess a cellular structure e.g., leaf, bark, petal, root etc or unorganized non cellular structural medicinal agents such as gum, latex etc. It may be a decoction which may be in cold water or prepared by bringing it to boil and allowing it to cool, or tisane which is tea made by either decoction, or infusion. Today, traditional medicine has brought to focus a wider coverage of primary healthcare delivery, not only in the African region but also, to various countries of the world. It is the first choice of healthcare treatment for at least 80% of Africans suffering from high fever and other common ailments (Elujoba, 2003).

Traditional medicine, a major African socio-cultural heritage, obviously in existence for several decades, was once believed to be primitive and wrongly challenged with animosity by foreign religions and conventional or orthodox medical practitioners (Elujoba, 2003). In recent years, natural products have aroused interest because drug resistance by diseases is on the increase and herbal remedies are being sought by a cross section of scientists for various ailments (White, 1997). Plants have always been considered to be a possible alternative and rich source of new drugs.

Phyllantus amarus is an important plant of Indian Ayurvedic system of medicine which is used for problems of the stomach, genitourinary system, liver, kidney and spleen (Patel *et al*., 2011). The plant has also been used in Brazil and Peru as a supposed herbal remedy for kidney stones. A clinical study with *Phyllantus amarus* indicated that it has no significant effect on either stone voiding or pain levels, but it may reduce the levels of urinary calcium.

Phyllantus amarus plant is used severally in some Northern and Eastern Nigerian communities for the treatment of fever (Dapper *et al*., 2007) and in some cases for the management of hypertensive cases. In a study conducted by Dapper *et al*. (2007) , the aqueous extract of *phyllantus amarus* was found to cause a significant dose-dependent suppression of *P-berghei* parasites in albino mice at doses of 108.33 165 and 325 mg kg^{-1} ($p<0.05$) in both early and established infection. In this study, the aqueous extract of *P. amarus* was evaluated for its effect on Percentage Packed Cell Volume (% PCV), Erythrocytes (Red blood cells) count, Leucocytes (White blood cells) count and body weights of *P. berghei*-infected mice treated with the extract. The partitioned fractions of the crude extract in hexane and ethyl acetate were also tested for antiplasmodial activities and the phytochemical composition of the crude was determined.

MATERIALS AND METHODS

Materials

Plant collection: Fresh leaves of *Phyllantus amarus* were collected from Bosso, Minna Niger State, Nigeria in the month of May and the plant was identified by the Botanist in the Department of Biological Sciences, Federal University of Technology, Minna, Niger State, Nigeria.

Experimental animals: This study were conducted in compliance with the Canadian Council on Animal Care (CCAC, 1997) to ensure humane treatment of all

experimental animals. Swiss albino mice of either sex weighing between 24-30 g obtained from Obafemi Awolowo University, (OAU) Ile-Ife, Nigeria were used for this study. They were fed with standard animal feed and water and maintained under standard conditions.

Parasite: *Plasmodium berghei* was obtained from National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria and subsequently the parasite was maintained in the Department of Biochemistry laboratory through weekly passage of infected mice blood into healthy mice.

Methods

Preparation of crude extract: The leaves of *Phyllantus amarus* were cleaned, dried and blended into coarse powder. Fifty grams of the powdered sample was soaked in 400 mL of water and refluxed for 2 h in a distillation flask mounted on a heating mantle according to the method described by Ugwu *et al.* (2011) and Kabiru *et al.* (2012). The extract was filtered hot using a cheese cloth and the filtrate evaporated using rotary evaporator and concentrated using water bath. The crude extract was weighed and stored in a refrigerator until required for use.

Partitioning of crude aqueous extract of *Phyllantus amarus*: Five gram of the crude aqueous extract was dissolved in 100 mL of n-hexane in a separating funnel and the mixture was shaken vigorously to ensure dissolution. The dissolved fraction was decanted into a beaker and the solvent allowed to evaporate leaving behind the semi-dried fraction. To the remaining residue in the separating funnel, 100 mL of ethyl acetate was added and shaken vigorously after which the dissolved fraction was decanted into a beaker and allowed to evaporate to obtain the semi-dried fraction. Both fractions were weighed and stored until required for use.

Parasite inoculation: The method of Kabiru *et al.* (2012) was used for the inoculation of parasites into experimental animals. The inoculums consisted of *Plasmodium berghei*-parasitized erythrocytes. Each mouse was inoculated on day 0, intraperitoneally with 0.2 mL of infected blood containing approximately 1×10^7 *Plasmodium berghei*-parasitized red blood cells. In addition, the newly inoculated animals were monitored daily to determine expression of parasites in circulation.

Monitoring parasitemia in suppressive test: It involved treatment with the extract immediately after mice have been inoculated (early infection). Fifteen male and female mice were divided into five group of three each. A mouse infected with *P. berghei* (parasitemia of about 20-30%) was anaesthetized with chloroform and sacrificed to collect the blood. The blood was diluted with normal saline such that 0.2 mL containing about 1×10^7 infected cells. Each of the fifteen mice was inoculated intraperitoneally with 0.2 mL diluted blood. The extract at dose levels of 200, 400 and 600 mg kg^{-1} body weight/day were administered orally to three test groups, respectively once daily for four days (Day 0, 1, 2 and 3). The positive control group was administered the standard antimalaria drug, Artesunate, at 50 mg kg^{-1} b.wt. The fifth group was left untreated and served as negative control. Similar groups were set up for the hexane and ethyl acetate fractions of the crude extract but the test groups were administered 50, 100 and 150 mg kg^{-1} body weight/day of the fractions.

The course of parasitemia was monitored using the method described by Mgbemena (2010) for four days. Percentage suppression of the parasite was calculated by the following formula:

$$\text{Suppression (\%)} = \frac{\text{Mean parasitemia in negative control-Mean parasitemia in treated}}{\text{Mean parasitemia in negative control}}\times100$$

Monitoring parasitemia in curative test: The inoculation and treatment procedures used for curative test was done according to the modified Peter's curative test (Peters, 1987).

This was similar to the suppressive test above except that treatment with the extract at 200, 400 and 600 mg kg^{-1} bodyweight of the extract and standard drug, artesunate at 50 mg kg^{-1} bodyweight commenced on Day 3 which is 72 h post inoculation and continued to Day 7. Same procedure was followed for the two fractions. Parasitemia was monitored on these days in order to determine percentage growth inhibition of the parasite.

Percentage growth inhibition of the parasite was also calculated the formula:

$$\text{Decrease in Parasitemia (\%)} = \frac{\text{Mean parasitemia in negative control-Mean parasitemia in treated}}{\text{Mean parasitemia in negative control}}\times100$$

Determination of hematological parameters: The parameters determined included, Percentage Packed Cell Volume (%PCV), Erythrocyte and Leukocyte counts.

Estimation of percentage packed cell volume (%PCV): Blood sample was collected from the tail of each mouse with a capillary tube by capillary action. The tube was sealed with plastestrine at one end. The sealed capillary tubes were then arranged on the haematocrit centrifuge and set to spin at 1200 revolutions per minute for five minutes. The % PCV was read on the haematocrit reader and recorded.

Determination of total leukocyte (white blood cell) count: This is the total number of white blood cell in the blood. This experiment was carried out using a haematocytometer-a device used for counting red blood cells and white blood cells in a sample of blood.

The blood was collected from the mice in an EDTA bottle to prevent clotting of the blood. Using the micropipette in the haematocytometer, the blood was taken to the 0.5 mark which was mixed with 0.85 mL of Gower solution. Using a hypodermic syringe, the blood mixture was placed on a haematocytometer slide to be viewed on a microscope with magnification of 40x. The White Blood Cell (WBC) count was noted and recorded.

Determination of total erythrocyte (red blood cell) count: This is the total number of red blood cell in the blood. This experiment was carried out using a haematocytometer-a device used for counting red blood cells and white blood cells in the blood.

The blood was collected from the mice in an EDTA bottle to prevent clotting of the blood. Using the micropipette in the haematocytometer, the blood was pipetted to the 0.5 mark which was mixed with 0.85 mL of Gower solution. Using a hypodermic syringe, the blood mixture was placed on a haematocytometer slide to be viewed on a microscope with magnification of 40x. The RBC was counted and recorded.

Phytochemical Screening of crude extract: The crude extract was subjected to phytochemical screening using the methods described by Sofowora (1993).

Data analysis: Data obtained in this study were analyzed using the statistical software SPSS 16.0, 2006 version (SPSS Inc, Chicago, Illinois, USA). Numerical data were presented as Mean±SD. The significance of the mean difference between two independent groups was determined using Student's t-test and one-way analysis of variance (ANOVA) while multiple comparisons were used when comparing more than two groups. A p-value<0.05 was considered significant.

RESULTS

Phytochemical compositions of crude extract of *Phyllanthus amarus*: The crude extract was found to contain flavonoids, saponins, phenols, alkaloids, tannins, reducing sugars and cardiac glycosides.

Suppressive antiplasmodial activity of crude extract: Figure 1 indicates that parasites were significantly suppressed in the groups treated with 200, 400 and 600 mg kg^{-1} body weight in a dose-dependent pattern. Though the highest suppressive activity was observed for the standard drug (Artesunate), there was no significant difference ($p<0.05$) between its activity and those of the crude extract given at 400 and 600 mg kg^{-1} bodyweight.

Curative antiplasmodial activity of crude extract: The curative activity was also dose-dependent for the extract as shown in Fig. 2. The standard drug-treated group however, showed the highest percentage decrease in the level of parasitaemia of treated mice.

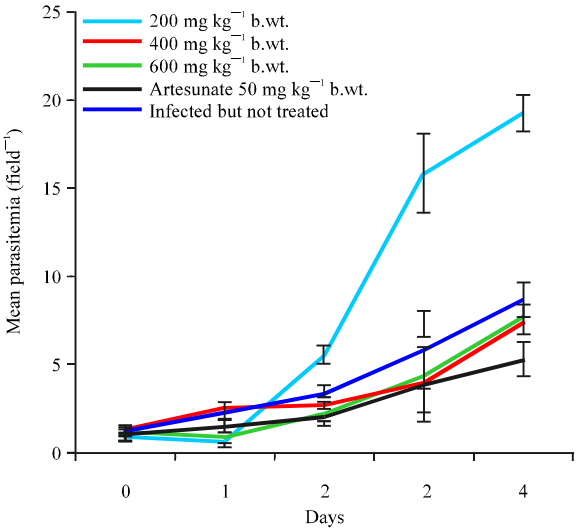

Fig. 1: Suppressive antiplasmodial activity of crude extract of *Phyllantus amarus* as compared to the standard drug (artesunate) and negative control in *Plasmodium berghei*-infected mice

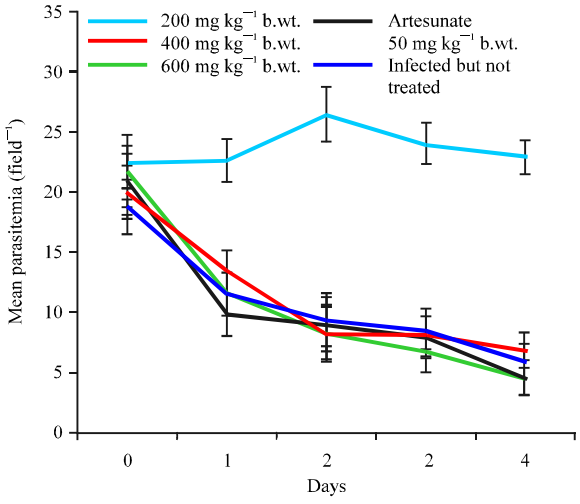

Fig. 2: Curative antiplasmodial activity of *Phyllantus amarus* after 72 h as compared to the standard drug (artesunate) and negative control in *Plasmodium berghei*-infected mice

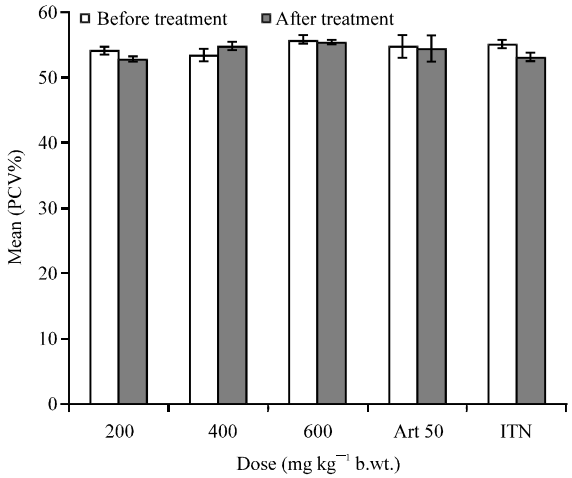

Fig. 3: Effect of crude extract of *Phyllantus amarus* on Percentage Packed Cell Volume (%PCV) before and after treatment for Suppressive group in *Plasmodium berghei*-infected mice. Art50 = Artesunate at 50 mg kg^{-1} b.wt. (positive control), INT = Infected but not treated (negative control)

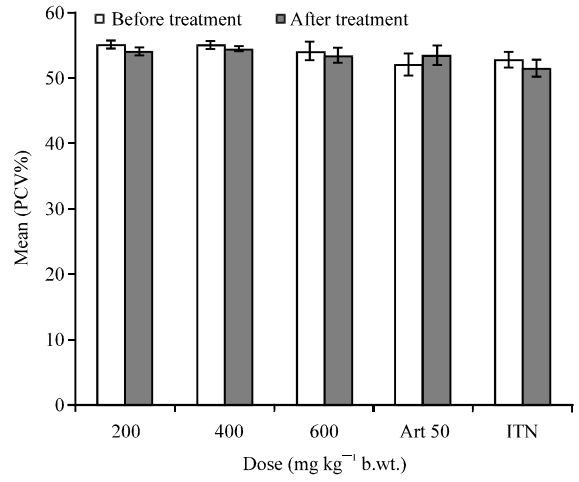

Fig. 4: Effect of crude extract of *Phyllantus amarus* on Percentage Packed Cell Volume (%PCV) before and after treatment for curative group in *Plasmodium berghei*-infected mice Art50 = Artesunate at 50 mg kg^{-1} b.wt. (positive control), INT = Infected but not treated (negative control)

Curative antiplasmodial activity of n-hexane and ethyl acetate fractions of the crude extract: The activity for both fractions was not significant compared to those of the crude extract and the standard drug ($p<0.05$).

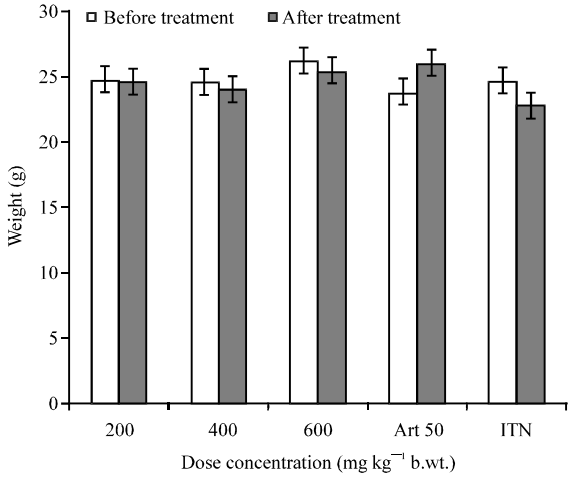

Fig. 5: Effect of crude extract of *Phyllantus amarus* on body weight before and after treatment for Suppressive group in *Plasmodium berghei* infected mice. Art50 = Artesunate at 50 mg kg^{-1} b.wt. (positive control), INT=Infected but not treated (negative control)

Effect of crude extract on percentage packed cell volume (% PCV) of infected and treated mice: Figure 3 and 4 shows the changes experienced in percentage packed cell volumes before infection and after treatment. There is no significant difference between the two sets of values ($p<0.05$) in both suppressive and curative groups.

Effect of crude extract on body weights of infected and treated mice: Figure 5 and 6 shows the changes experienced in body weights before infection and after treatment of mice. There is no significant difference between the two sets of values ($p<0.05$) in both suppressive and curative groups.

Changes in erythrocyte (RBC) and leukocytes (WBC) counts in infected and treated mice: Table 1 and 2 represent the values of erythrocytes and leukocytes before and after treatment of infected mice. There was no significant difference between the values of erythrocytes among the groups treated with the extract and the standard drug for both suppressive and curative groups at $p<0.05$. It was same for the change in leukocytes count except for groups H and I treated with 600 mg kg^{-1} body weight/day in the curative group where there was a significant difference in the values before and after treatment at $p<0.05$.

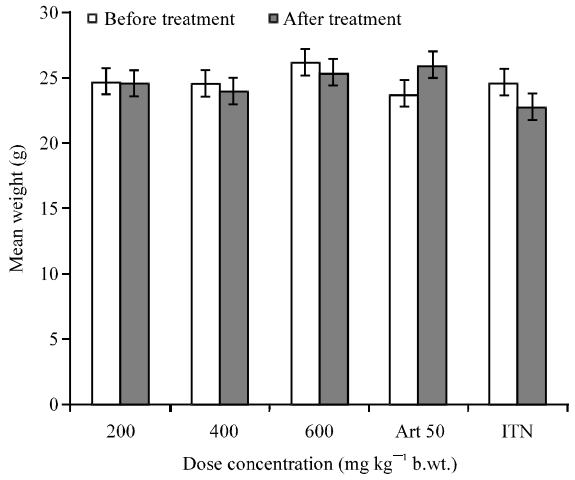

Fig. 6: Effect of crude extract of *Phyllantus amarus* on body weight before and after treatment for curative group in *Plasmodium berghei* infected mice. Significant difference at $p<0.05$. Art50 = Artesunate at 50 mg kg^{-1} b.wt. (positive control), INT=Infected but not treated (negative control)

Table 1: Erythrocyte (red blood cell) counts in infected mice treated with *P. amarus* crude extract

Group	Before treatment	After treatment
A	42.67±0.33	41.33±0.33
B	42.00±0.58	44.00±0.58
C	43.67±0.33	44.33±0.33
D	42.67±0.33	43.67±0.67
E	42.67±0.33	39.33±0.33
F	41.00±1.53	40.33±0.33
G	41.00±0.58	43.00±0.58
H	41.00±1.00	44.00±0.58
I	39.00±0.58	43.67±0.33
J	39.00±0.58	37.33±0.67

Groups A-E represent the suppressive groups while F-J represent the curative groups

Table 2: Leukocyte (white blood cell) counts in infected mice treated with *P. amarus* crude extract

Group	Before treatment	After treatment
A	11.33±0.33	11.33±0.88
B	11.33±0.88	10.67±0.33
C	12.00±0.15	11.33±0.88
D	12.00±0.15	10.67±0.33
E	12.33±0.33	13.67±0.33
F	14.00±0.58	13.67±0.33
G	14.00±0.58	11.33±0.88
H	13.67±0.33	9.33±0.33
I	14.33±0.33	9.67±0.33
J	13.67±0.33	14.00±0.58

Groups A-E represent the suppressive group while f-j represent the curative groups

DISCUSSION

The results obtained in this study has shown that what Mgbemena (2010) reported of the antiplasmodial activity of aqueous extract of *Phyllantus amarus* in *P. berghei*-infected mice is valid because the crude extract also demonstrated a dose-dependent anti-plasmodial activity for both suppressive and curative tests ($p<0.05$). There was a significant suppression of *P. berghei* in mice dosed with *P. amarus* but was more effective as a curative agent at 600 mg kg^{-1} body weight/day (Fig. 1, 2). However, the hexane and ethyl acetate fractions obtained from partitioning did not exhibit any significant antiplasmodial activities comparable to the crude extract. It is therefore, logical to infer that partitioning of the crude extract resulted in the loss of activity and that the crude extract has components that are acting synergistically to elicit antiplasmodial activity. It is conventionally expected that when crude extracts are fractionated, the purer fractions will elicit better efficacies, but surprisingly in this study, the hexane and ethyl acetate fractions did not exhibit appreciable anti-plasmodial activities. A loss of activity might have resulted from the fractionation due to loss of synergy. Similar cases of loss of activity due to fractionation have been reported. In a study conducted by Noedl *et al.* (2003), the fractionation of the methanol extract of *Eucalyptus camaldulensis* (leaf) resulted in decreased anti-plasmodial activity.

It has been suggested that crude extracts tend to have better plasmodistatic than plasmodicidal effects because some unpurified bioactive principles may require initial conversions which time lag allow for parasite proliferation (Noedl *et al.*, 2003). Moreover, the active components might not be present in enough concentrations to effect rapid and complete clearance of target organisms (Fidock *et al.*, 2004), as was observed in this study.

The effects of the crude extract on Percentage Packed Cell Volume (%PCV) is note worthy. The %PCV was seen to experience some changes after treatment (Fig. 3 and 4). This phenomenon is normal due to the effect of the extract on the cells in the blood especially the Red Blood Cells (RBCs) since the plasmodium parasites are usually localized in these cells and any treatment protocol will involve lysing of the cells and consequently this will affect the percentage packed cell volume. This condition is usually temporary because when the parasites are cleared from circulation, the cells begin to gradually divide and replenish the blood. The slight fluctuation in the erythrocytes counts (Table 1) is also understandable from the point of parasite lysing infected cells while the change experienced in the leukocytes counts (Table 2) may be due to the fact that they is responsible for defensive response of the body to disease because they help to boost the immune system of the body and scavenge parasite in the blood.

The antiplasmodial activities of several medicinal plants have been attributed to the presence of some of the phytochemicals like saponins and alkaloids identified in the crude extract of this plant (Kabiru *et al*., 2012). The phytochemical diversity of a plant species is indicative of its high medicinal and therapeutic potentials. This is because these compounds form the basis of the pharmacologic effects of such plants (Haidet, 2003). The crude extract of *Phyllantus amarus* was found to contain some of these phytochemicals, the presence of one or more of which may confer on the extract its antiplasmodial activity.

The change in the weights of infected mice is expected because malaria is characterized by loss in weight due to loss of appetite. But it was observed that the animals started to gain weight after the period of treatment (Fig. 5, 6).

It can be concluded from the results obtained in this study that the aqueous extract of *Phyllantus amarus* does not adversely affect the hematological parameters determined when used to treat *P-berghei*-infected mice.

ACKNOWLEDGMENTS

This study was funded by a research grant to Emmanuel O. Ogbadoyi by the Organization for the Prohibition of Chemical Weapons (Hague) and a Science and Technology Education-Post Basic (STEP-B) grant to the Federal University of Technology, Minna, Nigeria.

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