In Vivo Antiplasmodial Activity and Safety of the Aqueous Ethanolic Shoot Extracts of *Phyllanthus amarus* Schum. and Thonn

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ABSTRACT

Background: Monoherbal aqueous shoot extracts of *Phyllanthus amarus* Schum. and Thonn (Euphorbiaceae) is used in traditional medical practice in Tororo, Uganda to treat malaria fevers. Previous studies have demonstrated antibabesial, anti HIV, antibacterial, trypanosomal effects as well as antiplasmodial activity based on schizonts. However, there is hardly any study reporting the antiplasmodial activity on any part of *P. amarus targeting* trophozoites. **Objective:** To evaluate the *In vivo* antiplasmodial activity against trophozoites and safety of aqueous ethanolic shoot extracts of *Phyllanthus amarus* Schum. and Thonn. **Materials and Method:** Standard methods of extraction were used to obtain the crude extract as well as semi purified fraction from the study plant extract. Aqueous ethanolic shoot extracts of *Phyllanthus amarus* were tested for their *In vivo* antiplasmodial activity in *Plasmodium berghei* infected mice and evaluated by the standard 4- day suppressive test. Acute toxicity studies of *Phyllanthus amarus* was performed in at the Department of Pharmacology and Therapeutics – Makerere University College of Health Sciences using established methods. Results revealed *In vivo* antiplasmodial activity with good suppression activity ranging from 53.40% to 69.46%. In toxicity tests, no mice died within 24 hours of exposure to the test drug at room temperature even at 5000 mg/Kg indicating that it is nontoxic. **Conclusion:** Aqueous - ethanolic extract of *Phyllanthus amarus* shoot extract possesses useful *In vivo* antiplasmodial activity against trophozoites when used at doses that cause no marked toxicity in mice.

INTRODUCTION

Herbal use for primary health care in addressing infectious tropical diseases like malaria is gaining recognition worldwide. The focus of this study was on malaria, a Protozoan parasitic disease which continues to devastate most people in the world accounting for over 216 million malarial cases with 445,000 deaths [1]. More than 90% of these cases occurred in sub Saharan Africa (SSA) with approximately 91% of all the malarial deaths [1].

More than 90% of all the malaria mortalities are caused by *Plasmodium falciparum* which is the most lethal species in humans [1].

Despite the fact that a lot of studies have been and/ are being carried out regarding malaria, it is still imposing an intolerable burden to the vulnerable group mostly on children under five years and pregnant mothers living in underserved rural areas [2]. According to this same WHO, malaria killed about 285,000 children under five years from sub-Saharan Africa alone [2]. [3] Malaria kills mainly children below five years of age because their immunity to it is still undeveloped by this age [4].

Moreover, the World Health Organization estimated that 2.7 Billion United States dollars was required in the malaria control and prevention programs globally and yet the majority of the countries most affected by the disease could only afford 31% of this [1].

For Uganda as a country, a recent Malaria Bulletin of 2016 reported that malaria is currently responsible for more illness and death than any other single disease in Uganda [5]. A significant heterogeneity of malaria transmission has been observed in Uganda where it has been shown that parasite prevalence among children under 5 ranged from less than 5% in Urban areas to more than 60% in the rural settings [6].

Malaria accounts for over 27 % of all deaths in Uganda [5], with over 90% of malaria cases being caused by *Plasmodium falciparum* (WHO, 2017) which represents a serious and increasing world public health problem due to the acquired resistance to most of the commonly available drugs. Realizing that there are ever increasing incidences of multi-drug resistant Plasmodium, there is a need to look for a new anti-malarial agents that are efficacious, affordable, safe and routinely available to people particularly those in resource limited countries. Thus, the communities in endemic areas have not only started using but are also looking out for malaria remedies in natural products [7].

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Plants have always been considered to be a possible alternative and rich source of the new drugs [8] for instance antimalarial drugs, like quinine and artemisinin were either obtained directly or indirectly from botanicals/by chemical structures of plant-derived compounds as templates. The extracts of a large number of plant species including many that are employed in traditional medicines have been evaluated for both *In vitro* and *In vivo* antiplasmodial activities [9].

In some cases, the constituent(s) responsible for their activities have been isolated but relatively few have been studied further to assess their potential as lead compounds for the development of new products to treat malarial.

In countries like Uganda where malaria is endemic, communities have frequently depended on herbal products to treat malaria[10]. The analysis of traditional medicines that are employed for the treatment of malaria represents a potential for discovery of lead molecules for development of antimalarial drugs[11]. A very good example that quickly comes to mind is that of quinine derivatives obtained from the bark of Cinchona tree of South America [12]. The recently formulated potent and effective artemisinin derivatives are isolated from Artemisia annua, a plant used for thousands of years to treat malaria by the Chinese people[13]. The success in isolation of artemisinin has inspired many researchers to look for new antimalarial drugs from plants that are being used to treat malaria in traditional healthcare systems. A baseline study we carried out in 2010, in Tororo, Eastern Uganda, revealed *Phyllanthus amarus* Schum. and Thonn.(Euphorbiaceae) as one of the important herbs used by Tororo communities as monoherbal preparation to treat malaria [14].

In Ghana, one of the medicinal plants used to treat malaria include *Phyllanthus amarus* (Euphorbiaceae, Schum. & Thonn.)[15]. Furthermore, *P amarus* is commonly used in Southeastern Nigeria for the treatment of malaria-related symptoms [16].

 Results of a phase two clinical trial conducted on antiplasmodial herbal remedy, consisting of eight ingredients including *Phyllanthus amarus* and administered by Nigerian herbalists, confirmed the efficacy of the remedy[17].

This wonder herb has been shown to inhibit the DNA polymerase of hepatitis B virus and reverse transcriptase of HIV[18]–[20].

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Various methods including *In vitro* and/or *In vivo* assays are employed in screening for new antimalarial drugs. *In vitro* antiplasmodial activity of *Phyllanthus amarus* against chloroquine sensitive *Plasmodium falciparum* strain 3D7 has been reported [16]. However, the *In vitro* test alone does not give a complete picture of the antimalarial activity as it does not cater for prodrug principles that can only be detected during the I*n vivo* screening hence the need for the latter. Although, anti-plasmodium effects of *P. amarus* of the genera Phyllanthus has been evaluated for anti plasmodium activity by microscopic based on schizonts [7], limited activity on any part of the monoherbal preparation of this wonder herb based on trophozoites have been reported for *in vivo* studies.

This study was, therefore, an effort to investigate the antimalarial activities of the monoherbal ethanolic extract of *P. amarus* against *P. berghei* using the four-day suppressive *in vivo* ant malarial test in order to authenticate its use in traditional medicine.

MATERIALS AND METHODS

Plant materials and extraction: The shoot of *P. amarus* was collected from Kamuli Village, Pagoya LCI zone -10 Km from Tororo, Town in Eastern Uganda (Fig.1), during the months of February 2010. The identification and authentication of the plant specimens was done at the Department of Botany Makerere University, Kampala where this species was previously collected and voucher specimens deposited.

Samples were air-dried at room temperature under shade, and ground into powder using an electric mill. The crude extracts were prepared by cold maceration technique[21].

The extraction was done by transferring 50 g of plant material into the distillation flask to which 300 ml of aqueous ethanol was added. The mixture was placed on an orbital shaker GLF 3020, Germany) at 100 rpm (room temperature) for 48 hours. The mixture was first filtered using cotton and then the filtrate was passed through Whatman filter paper (No.3, 15 cm size with retention down to 0.1μm in liquids). The Merck was washed three times with ethanol (20ml X 3) to exhaustion.

The ethanolic extracts were concentrated in a rotary evaporator (Buchii type -114, Germany) at a temperature of 45°C to afford 5g of the crude extract (10%) – used for the antimalarial assay and acute toxicity tests. For the antimalarial assays, a little methanol was added to

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dissolve the residue which was then transferred to a Petri dish which was placed into the Vacuum chamber to dry gradually in readiness for the antimalarial assay.

Animals: Male Swiss albino mice were provided by the Department of Biology, Faculty of Science- Addis Ababa University (AAU). They were housed in standard cages being given standard feed and water *ad libitum*.

All the animal experiments were performed according to the guidelines for animal experimentation – department of Biology, Addisa Ababa university- Addisa Ababa, Ethiopia.

The parasite and infection: For *In vivo* anti-malarial assays of plant extracts, Chloroquine (CQ) sensitive strain of *P. berghei* maintained at the animal house of the Biology Department, Addis Ababa University was used. The parasites were maintained by serial passage of blood from infected mice to non-infected ones on weekly basis.

To infect the mice, blood sample was collected from auxiliary vessels of a donor mouse with a rising parasitaemia of about 30-37%. Then, the blood was diluted in normal saline so that each 0.2 ml contained approximately 10^6 infected red blood cells. Each animal received inoculums of about 10^6 parasites via intraperitoneal route (IP). The inoculated mice were then randomized into four mice per cage and maintained in the animal house on a commercial diet and water ad libitum.

Anti-malarial activity of plant extracts: In screening of the plant extracts, the standard four-day suppressive method was used[22]. Male Swiss albino mice aged 6-8 weeks and weighing 26-32g (Ghosh 1984) were infected with10^6 *P. berghei* and randomly divided into five groups of four mice per cage. The infected mice were randomly divided into three test groups and two control groups each (for CQ as a standard drug and 3% Tween 80 as a negative control).

The test extracts were prepared in three different doses (250mg/kg, 500mg/kg, and 1000mg/kg of body weight) and CQ at 25mg/kg in a volume of 0.2ml and vehicles at 0.5ml/mouse. Each dose of the extract was administered as a single dose per day.

All the extracts and the drug were given through intragastric route by using standard intragastric tube to insure safe ingestion of the extracts and the standard drug (chloroquine).

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Treatment was started after 3 hrs of infection on day 0 and was then continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D4) blood sample was collected from tail snip of each mouse. Thin smears were prepared and stained with 10% Geimsa solution.

Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups. The experiments were done by the author, the parasite count was performed by an experienced technician (double blinded) and the study animals were followed for up to 28 days to monitor survival rates post dosage.

The numbers surviving per group was recorded at day 7, 14, 21 and 28 from the first dose (Day 0). Results of this follow up are summarized in figure 2. The animal keeper looked after the experimental mice during the course of the study.

Percentage parasitaemia was calculated from the results of the microscopic examination.

Acute toxicity Studies

Acute toxicity Studies of *Phyllanthus amarus* was performed in the animal house located at the Department of Pharmacology and Therapeutics – School of Biomedical Sciences, Makerere University College of Health Sciences in January 2012.

Using established methods[23], ethanolic- *Phyllanthus amarus* shoot test extracts were prepared at dose levels of 0, 200, 600, 2000 and 5000mg/Kg body weight.

Swiss albino mice aged 6-8 weeks and weighing 26-32g [23], maintained at the animal house located at the Department of Pharmacology and Therapeutics – School of Biomedical Sciences, Makerere University College of Health Sciences from 2nd January 2012**.** Both male and female mice were randomly divided into three test groups and 1 control group each with a total of 10 mice.

Prior to undergoing the acute toxicity test, all the test mice were fasted for 18 hours followed by the treatment group receiving 200, 600, 2000 and 5000mg/kg while the negative control group was administered 10mg/Kg of Isotonic saline. All the extracts doses were given through intragastric route by using standard intragastric tube to insure safe ingestion of the extracts and the isotonic saline.

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All the mice treated with the test drug and isotonic saline were then maintained in the animal house on a commercial diet and water ad libitum.

Close observations 2 hours, 4 hours, 6 hours and overnight post administration (monitoring for the behaviour of the animal including convulsions in mice observed as tremor in the tail and/paddling of the feet including cardiac arrest and mortalities within 24 hours post administration. Results of this study are presented in Table 2.

Ethical Consideration

Experimental protocols approved by the Higher Degrees Committee and Uganda National Council for Science and Technology (NO.HS 706) were used according to the internationally accepted standard guidelines for Care and Use of Laboratory animals [23]. All animals used in this Experiment were handled with utmost human care- with no pain and cruelty [23].

Data analysis

Results of the study were expressed as mean ± standard error of mean (M ± SEM). Comparison of parasitaemia and statistical significance was determined by Kruskal-Wallis Test and post hoc Wilcoxon Rank Sum test (Dawson and Trapp 2004; Longnecker and Ott 2001) test using Stata Version 9 for windows statistical package. All data were analyzed at a 95% confidence interval (P =0.05).

Percent parasitaemia and percent suppression were also calculated as follows:

% parasitaemia per group = (Average No. of infected RBCs/Total No. of RBC)*100.

On the other hand, percent suppression (%S) was determined by:

%S = [Parasitaemia in the negative control (NC) – parasitaemia in the test group /parasitaemia in (NC).These results are summarized in table 1.

RESULTS OF THE STUDY

Demography

Kamuli village (Numbered 2 in the map) is in Mukujju-one of the Sub counties of Tororo district in Uganda (shown in figure 1). Mukujju has an approximated population of 37,749

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with a sex ratio of 82.5% that is 82.5 males per every100 females(Tororo district planning unit 2009/2010).
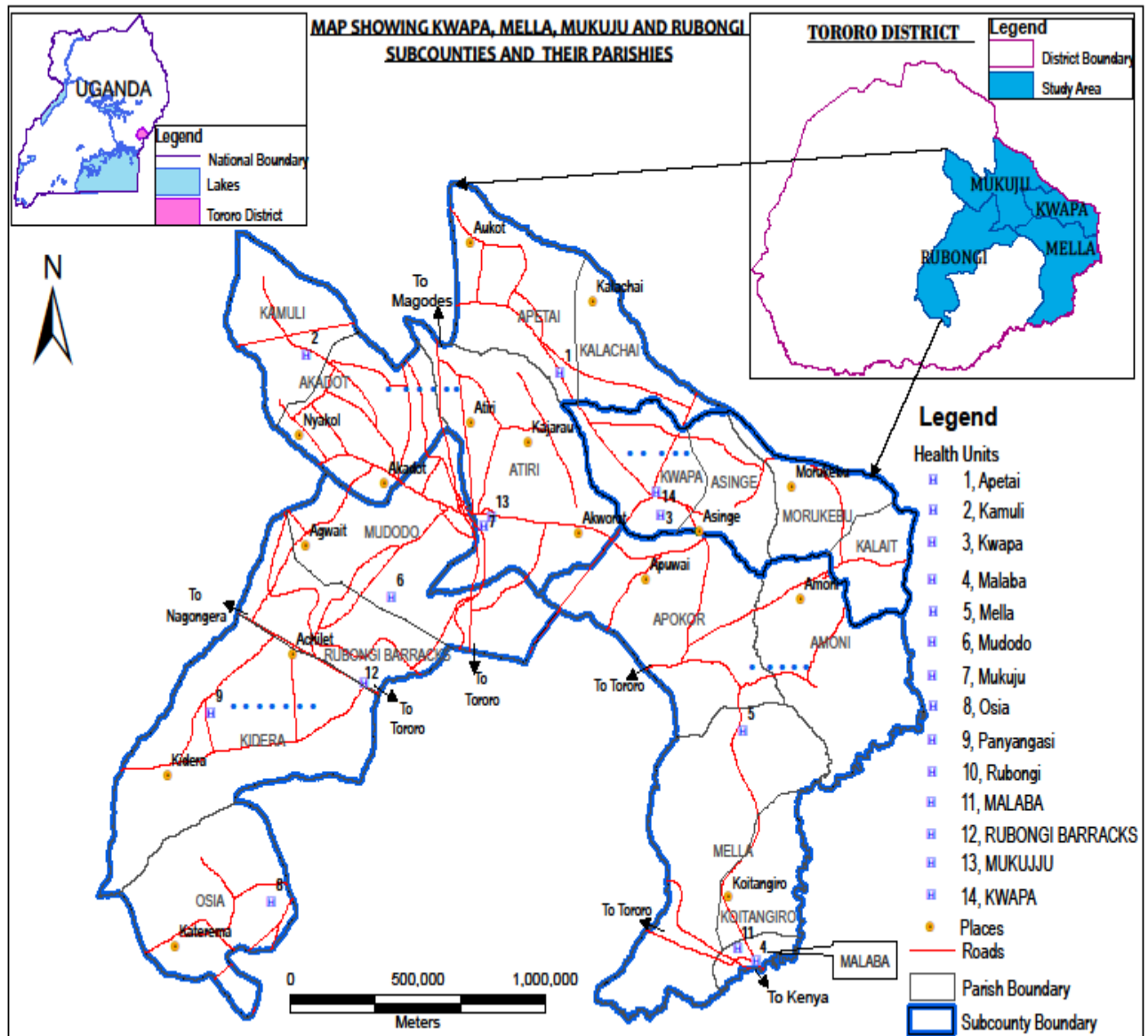

Figure 1: Map of Tororo District in Eastern Uganda – showing Kamuli Village in Mukujju Sub-county - the source of *P. amarus*

RESULTS OF ANTIMALARIAL ACTIVITY

The results of the *In vivo* antimalarial tests are summarized in table 1. The *P. berghei* mice model was used since it is the first recommended step to screen most of the *In vivo* anti-malarial activities of new molecules and new therapeutics [24].

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The ethanol extract of *P. amarus* showed 55.3 % suppression of parasitaemia at the dose of 250mg/kg (Table 11).

Table 1: Activity of ethanolic extract of the shoot of *P. amarus* against *P. berghei* in mice

Extract	Dose (mg/Kg/day)	Anti-malaria activity	
		% Parasitemia ±SE	% Suppression
02-1C	NC	42.3±0.16[a]	0.00%
Group 1(GP 1)	250	18.9±10[a]	55.30%
Group II (GP II)	500	12.4±7[c]	70.70%
Group III (GP III)	1000	0±0.0[d]	100.00%

Values are mean ± SE, n = 4 except in 250mg/Kg dose group where 1 mouse died on day 2 of the treatment process; NC: Negative control (0.5mL vehicle); a, b, c.d) = values in the same column followed by the same letter do not differ significantly.

The highest suppression of parasitaemia was observed at the dose of 1000mg/kg body weight of mice. Percentage suppression was observed to increase as extract dose increased.

After four days treatment with the different extract doses, the mean parasitaemia of the test groups ranged from18.90±10 to 0.00± 0.0% while the corresponding value of the negative control (NC) group was 42.3±.16 %.

The mice treated with 1000mg/Kg dose of the test extract was the same as for those treated with 25mg/Kg chloroquine – the positive control (PC) and were completely free from the parasites on day four.

Significant reduction of parasitaemia (P < 0.05) was also observed in all groups of mice treated with ethanolic extract of the shoot of *P. amarus* compared to the negative control (NC) except the 250mg/Kg group. Figure 2 is a summary on following up the groups (NC, PC, Group I, Group II and Group III) for any signs of reoccurring malaria. Results in Figure 2 show the percent survival of the subjects over 28 day period.

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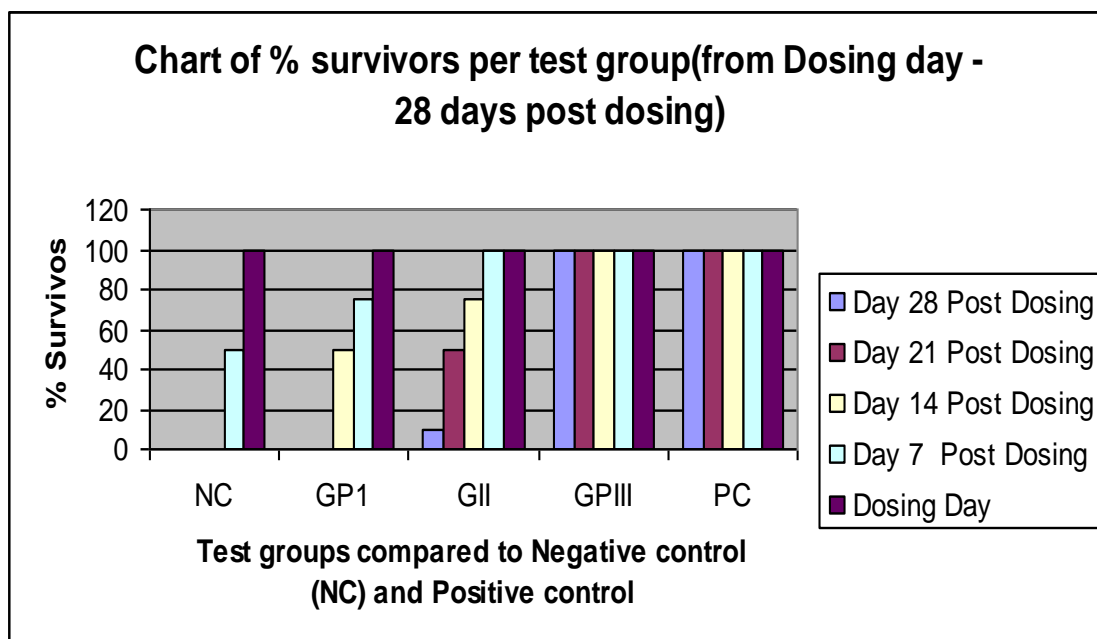
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Figure 2: Percentage of survivors in each test group (group 1-III, Negative control and Positive control-PC) from initial dosing day to 28 post initial dosing

These results show that there was no recrudescence in the mice treated with 1000mg/Kg dose of the test extract whose % parasite suppression was the same as for those treated with 25mg/Kg chloroquine and that the mice were completely free from the parasites infection.

RESULTS OF SAFETY (ACUTE TOXICITY) STUDIES

In the toxicity tests, all the mice administered ethanol- *Phyllanthus amarus* shoot test extracts at dose levels of 200, 600, 2000 and 5000mg/kg exhibited insignificant signs of toxicity, ranging from writhing and gasping. No mice died within 24 hours of exposure to the test drug at room temperature even at 5000mg/Kg dose (LD50 of >600 mg/kg), indicating that it is nontoxic table 2.

The present results indicate that the ethanolic extract of *Phyllanthus amarus* shoot test possesses useful anti-malarial activity (against trophozoites) when used at doses that cause no marked toxicity in mice. Although the mechanism of action of this compound has not been elucidated, ethanolic extract of *P. amarus* shoot clearly merits further investigation.

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Table 2: Results of the safety (Acute toxicity test) of the various dosage levels of ethanolic extracts of *P. amarus* shoot on Swiss Albino mice after a 24 hour incubation at room temperature

Dose Mg/Kg	Log10 Dose	No. of mice tested /dose/cage			No. of mice dead after 24 hour exposure to drug and negative control			% Mortality
		Male	Females	Total	Males	Females	Total	
0		5	5	10	0	0	0	0
200	2.3010	5	5	10	0	0	0	0
600	2.7782	5	5	10	0	0	0	0
2000	3.3010	5	5	10	0	0	0	0
5000	3.6990	5	5	10	0	0	0	0

DISCUSSION

Phyllanthus is a well-studied genus especially for antiplasmodial activity because of its traditional use against malaria in other endemic regions [15], [7]; [25].

Results of a phase two clinical trial conducted on antiplasmodial herbal remedy, consisting of eight ingredients including *Phyllanthus amarus* and administered by Nigerian herbalists, confirmed the efficacy of the remedy[17].

This study was designed to assess the *In vivo* antimalarial activities of aqueous ethanolic *Phyllanthus amarus* shoot crude extracts processed by indigenous knowledge.

The *In vivo* antiplasmodium effects of *P. amarus* of the genera Phyllanthus has already been evaluated by Dapper *et al*., 2011 with microscopic examination based on schizont [7]. However, there is hardly any report on the *In vivo* antiplasmodial activity on any part of *P. amarus* based on trophozoites. The WHO malaria control unit considers antimalarial treatment satisfactory if total clearance of parasitaemia (trophozoites) is within 7 days with the diseases not reoccurring within 28 days of the initiation of the treatment.

Moreover, *In vitro* testing alone cannot be used to predict a patient's clinical response to treatment as it may fail to detect antiplasmodium principles which act in the same manner as

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prodrugs. Antimalarial prodrugs undergo enzymatic transformations *In vivo* to give active antiplasmodial compounds [26].

Therefore there was a need to carry out an *In vivo* testing based on trophozoite counts other than Schizont counts(WHO).

The ethanolic extracts of the shoot of *P amarus* was tested for its antimalarial activity against *P. berghei* in mice. The antimalarial activity of the ethanolic extracts and their respective interpretations were made in accordance to [27]. WHO classified the *In vivo* antimalarial effect of botanicals as follows: at a dose of 1000mg/kg/day, when the % inhibition is higher than 50%, the activity is considered moderate; otherwise it is considered inactive[27]. This dose is made use of to detect if an active product is present in only small amounts in the extract.

At a dose of 500mg/kg/day, when the % inhibition is equal to or higher than 50%, the activity is also considered moderate; at a dose of 250mg/kg/day, when the % inhibition is equal to or higher than 50%, the activity is considered good.

The ethanolic extract of the shoot of *P. amarus* showed good antimalarial activity and significantly inhibited parasitaemia of *P. berghei* in mice (p< 0.05). The activity values obtained in this study are comparable to those of known plant remedies of various origins [21] [28].

The suppression activity of this extract was observed to be dose dependent, increasing with increase in the concentration of the extract compared to the negative control.

The *P. berghei* mice model was used since it is the first recommended step to screen most of the *In vivo* anti-malarial activities of new molecules and new therapeutic [24], [29].

Furthermore*, P. berghei* is recognized as valuable model organism for the investigation of malaria in humans because they are similar in most essential aspects of morphology, physiology and life cycle and the manipulation of the complete lifecycle of these parasites, including mosquito infections, is not only simple but also safe.

It is similar to other malaria parasites affecting mammals including the four malaria parasites affecting man, in that *P. berghei* is transmitted by the bite of Anopheles mosquitoes and it infects the liver after being injected into the bloodstream. After a few days of development

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and multiplication, these parasites leave the liver and invade red blood cells. The multiplication of the parasite in the blood causes the pathology such as damage of essential organs of the host such as lungs, liver, spleen and anemia. *Plasmodium berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice [30]; [31].

Phyllanthus amarus has widely reported uses for various ailments:(Kiritikar and Basu, 2001) reported expectorant, diaphoretic and diuretic activities from the leaves of *P. amarus*. Studies by Syamasundar and friends demonstrated the hepatoprotective activity of Phyllanthin and hypophyllanthin present in *Pamarus* [33].

Furthermore, Thyagajaran and colleagues in1988 and Blumberg *et al*.,1990 reported that *P. amarus* exhibited significant *In vivo* anti-hepatitis B virus Surface antigen activity [34]. The *In vitro* activity against the same was reported by Mehrotra and friends in 1991[35].

Furthermore, this wonder plant has been reported exhibiting anti-fungal (Huang *et al*.,2003), Liver protective and detoxification, [36],anti–cancer, (Kumar and Kuttan,2005). However, of particular interest was that no published scientific report of it's *In vivo* anti-malaria activity based on trophozoites has been reported as required by WHO yet local communities in Tororo district, Eastern Uganda use it for this purpose. This study was, therefore, an effort to address this gap.

The parasitaemia suppression effect of the extract may be attributed to the presence of Flavonoids, Quercetin, anthraquinones, triterpenoids present in Phyllanthus species [37], [38], [39]. It is possible that these bioactive compound(s) found in the shoot of this plant could probably have resulted from a single or combined effect of these compounds. The antimalarial activity of quercetin was reported by [37], [38], [39].

Finally, it is important to note that one mouse died on day 2 of the treatment, reducing the group to 3 instead of 4 as in the other groups and this could have had an effect during the calculation of the average parasitaemia in the group compared to if the number was 4 as in the other groups.

Therefore, this study, together with the aforementioned reports, shows that the plant may serve as a potential source of different anti-plasmodial compounds.

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CONCLUSION

The crude extracts of *P. amarus* shoot exhibited significant antimalarial activities thus indicating their positive role, and justifying the ethnomedicinal use of the plant parts in traditional medicine.

Furthermore, the ethanolic extract of *Phyllanthus amarus* shoot possesses useful antimalarial activity (against trophozoites) when used at doses that cause no marked toxicity in mice.

ACKNOWLEDGMENTS

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