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EVALUATING IN VIVO ANTIPLASMODIAL POTENCY OF AQUEOUS EXTRACT OF PHYLLANTHUS NIRURI IN SWISS ALBINO MICE

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[Ω]To a fond memory of Prof. V.A. Togun who passed-on in course of this study. R.I.P.

ABSTRACT: *In vivo antiplasmodial potency of aqueous leaf extract of phallanthus niruri used in malaria naturopathy, a global public health scourge was evaluated in unsexed 100 4-week old, 5 groups of 20 swiss albino mice each weighing 18-22g passaged with rodent plasmodium berghei NK-65 except Sham. Control/Sham received distilled water. Other 3 groups received 200, 400; 800mg/kg of extract respectively and focally/histologically sampled for 7 consecutive days (d). After 7d, Control exhibited high density load of parasitemia, marked lethargy, white eyelids and extreme hemodilution compared to treated groups. % residual parasitic density with time (d) were 35 ± 4% (200mg/kg), 13 ± 1% (400mg/kg), 10 ± 1% (800mg/kg) compared to 65 ± 7% (Control) with Ct½ 1.4 < 2.3 < 4.8 < 7d : 800 < 400 < 200mmkg < Control ($p < 0.05$). No adverse renal or hepatic effects. These results strongly demonstrate a potent safe in vivo antiplasmodial activity of P. niruri.*

KEYWORDS: antiplasmodial activity, phyllanthus niruri, aqueous leaf extract, swiss albino mice.

INTRODUCTION

Malaria continues to be a major cause of morbidity and mortality in many tropical and sub-tropical countries. Various estimates by the World Health Organization (WHO) put up to 80% of death occurring in Africa South of Sahara to be due to malaria. In addition to acute disease episodes and deaths in Africa, malaria also contributes significantly to anaemia in children and pregnant women. Adverse birth outcomes such as spontaneous abortions, still births, premature deliveries and low birth weight, and overall child mortality are consequent upon these episodes. Economically, the disease is estimated to be responsible for an estimated average annual reduction of 1.3% in economic growth for those countries with higher malaria burden (Sachs, 2001). In Nigeria for example, aside from ravages of the adults population, malaria is responsible for death and morbidity amongst infants below the age of 5 years (Agomo et al., 1998). It accounts for 25% of under 5 year infant mortality and 30% overall childhood mortality and 11% maternal mortality. Over 86% of these under-5 year children's

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illnesses are due to malaria (FMOH, 1991). These foregone statistics raise malaria enigma to the forefront of global medical research potential.

Medicinal plants have been used in traditional medicine for hundreds of years in many countries of the world (Oubre et. al, 1970). This is in line with the various WHO declarations that medicinal plant researches warrant attention. And naturopathy has become recognized as alternative therapy. Africa has arguably one of the richest phyto-diversities in the world. Africa forest geographically spans approximately 216,634,000ha (Farombi, 2003). More than 50% of all modern clinical drugs are of plant origin and natural products play an important role in drug development programs of the pharmaceutical industry (Baker et al., 1995). In malaria endemic countries, traditional medicinal plants are frequently used to treat malaria especially in rural and sub-urban settings (Nondo et al., 2016). The analyses of traditional medicines that are employed for the treatment of malaria represent a potential in the global efforts at discovery of lead molecules for development of antimalarial drugs. For example, quinine derivatives were modeled on the quinine molecule, found from the bark of Cinchona tree of South America (Garnham, 1966). The recently formulated potent and effective artemisinin derivatives were isolated from Artemisia annua, a plant used for thousands of years to treat malaria by the Chinese people (Mueller et al., 2000). Due to this success, many researchers are now interested in looking for new phyto-antimalarial drugs. Phyllanthus niruri (P.niruri) has many effective traditional uses for a wide variety of diseases. Some of the medicinal uses have been supported in experimental models across the globe, suggestive that the plant extracts possess various pharmacological properties and hence the biotic basis of most pharmacopias (Patel et al., 2011; Vermad et al., 2014).

Given the aforementioned facts and the claim use of these plants for malaria control and a further elucidation of P. niruri anti-malaria activities, this study has thus in a randominized design, evaluated the antimalarial activity of aqueous leaf extract of P. niruri in an in vivo system of Swiss mice model, a closer phylogenetic relative of man of all the rodents species models with a relative applicability. Renal and hepatic functions of these animals were also evaluated to verify the safety of the extracts per OS administration.

MATERIALS AND METHODS

Plant Collection, Authentication and Extraction

The plant P.niruri was collected from its natural hahitat in and around Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Oyo State, Nigeria. It was taxonomically identified and authenticated by an Angiosperm Taxonmist in the Biology Department, LAUTECH, Ogbomoso, Oyo State, Nigeria and issued a voucher number, LH0374. A specimen of the plant was deposited at LAUTECH Herbarium, Ogbomoso, Nigeria. The herb was air-dried and pulverized to fine powder. A 200g portion of the powder was carefully measured out and soxhalated with 1000ml of water as solvent for 24 hours (h), followed by evaporation under reduced pressure to obtain the aqueous extract.

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Animal

Hundred (100) unsexed 4 week-old-swiss albino mice weighing 18-22g were obtained from the Animal Facility Centre of National Agency for Food and Drug Administration and Control (NAFDAC), Yaba, Lagos, Nigeria. Upon arrival they were initially housed in standard aluminium cages with saw dust beddings for an initial 7d acclimatization period. Feed and water were given ad libitum. The mice were used in accordance with NIH Guide for the use and care of laboratory animals; NIH Publication (No. 83-23) revised (1985).

Rodent Parasite (Plasmodium berghei)

The rodent parasite Plasmodium berghei NK 65 was obtained from The Nigerian Institute of Medical Research (NIMR),Yaba, Lagos, Nigeria and kept at their Animal Facility Centre. The parasites were kept and maintained alive by continuous serial intraperitoneal passages in these donor mice (Adzu and Haruna, 2007) with a 4 day cycle. These infected mice were used as donor mice for the study. Prior to the beginning of the study, a small group of the infected mice was retained and observed to reproduce disease symptoms similar to human infection (English, 1996).

Curative Treatment Test

This model of malaria treatment in experimental mice was carried out basically according to the methods of Ryley and Peter, 1970. Essentially, following the initial acclimatization period, all animals were divided into 5 groups of 20 mice each. All the animals except Sham were then passaged with malaria parasite in phosphate buffered saline (PBS) intraperitoneally in a total final volume of 0.1ml from donor mice, and left for a further 72h for the parasites to manifest, before the commencement of the treatment. Thereafter, the extracts were administered to each animal in a total final volume range of 0.3ml - 1.4ml depending on extract dose. The Sham was passaged with parasite bed-volume of PBS. Group I (Control) and II (Sham) mice were given daily dose equivolume of vehicle, distilled water per kg by gavage. Groups III, IV and V were given 200, 400 and 800mg/kg (0.3, 0.6 and 1.4 final volumes) of extract each respectively. The animals were treated for 7 consecutive days from the commencement of treatment at the day zero (D0), ie (D0, D1, D2, D3, D4, D5, D6, D7). All animals were focally sampled and parasitemia was monitored histologically by blood smears of each mice everyday for 10 days.

Focal Sampling

All animals were focally sampled by timed observations as modified from Okwusidi (1988). Briefly, each animal (or animal pair) was observed for 10-20minute (m) periods: the Control for parasitemia associated lethargy and other behavioral deficits. The extract administered animals were likewise focally sampled for behavioral effects associated with experimental manipulations and extract (drug) effects.

Microscopic diagnosis of malaria parasite

Thick and thin blood films were made with free-flowing whole blood directly from the mouse tail snips for histologic evaluation. The thick film was not fixed to allow lysis of the RBCs during staining. The thin films were used for qualitative and quantitative determinations or confirmation of the plasmodium species if necessary in routine examination and in mixed infections. The thin film was fixed with absolute methanol to allow the parasites morphology to be seen within the RBCs by giemsa staining technique. Up to 100 fields of the blood films were subsequently examine in a zig-zag pattern to determine whether the blood film was positive or negative for malaria parasite. Parasites were quantitatively determined per ml of blood according to equation:

$$\text{Parasites per microlitre} = \frac{\text{number of observed parasites}}{\text{Number of leukocytes}}\ x\ 8000 \qquad \text{(WHO, 1991)}$$

Hepatic and Renal Functions Test

The functional status of the liver and kidney were assessed by measuring their function test indicator enzymes, serum aspartate transferase (AST) and alanine transferase (ALT) for the estimation of hepatic patency (Okwusidi, 2015) and blood urea nitrogen (BUN) for estimation of non-specific kidney damages and serum creatinine (CR) measurements for specific damange to the kidney (Panda, 1999; Okwusidi, 2015a, Okwusidi & Togun, 2016). Commerical test kits from Randox Laboratory were used to evaluate AST, ALT, BUN and Serum CR. All the test were monitored spectrometrically at a wavelength of 546nM.

Experimental Design and Statistical Analysis

The experimental design and treatment regime are outlined in Table 1, showing sequence of receipt and non-receipt of treatment by study animals.

Table 1: Experimental design/protocol and sequence of treatment regimen showing animals receiving (+) and not receiving (–) treatment

GROUP	TREATMENT				
	Parasite	Distilled Water	Extract (mg/kg/d)		
			200	400	800
I	+	+	–	–	–
II	–	+	–	–	–
III	+	–	+	–	–
IV	+	–	–	+	–
V	+	–	–	–	+

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The experimental design was a completely randominized design (CRx). All values of measured variables were reported as mean ± standard error of the mean (SEM). Data were analyzed by analysis of variance (ANOVA) employing the statistical analytical software/package (SAS). Duncan Multiple Range Test was used as post hoc test of significance to separate all significant means. A $p < 0.05$ was considered statistically significant (Daniel, 1983; Godfrey, 1985).

RESULTS

Evident from focal sampling, the mortality rate and the acute toxicity signs were manifest and increased with time only in the distilled water treated parasitized control group which were not administered any aqueous extract of P. niruri. The main observed behavioural signs of toxicity were whitish eyelids, lethargy/sluggishness, weakness and death. These animals exhibited a relative high death to treatment (D/T) ratio, with a latency (time to death (d) post parasitemic innoculation) of 4d. Death possibly due to the shock of the accumulated high density load of the parasites in the blood of the mice. These parasitized control animals exhibited extreme hemodilution and a lowered threshold to hemolysis. In contrast, the Sham and P. niruri treated mice did not exhibit any of the behavioural debilities observed in the Controls or untoward reactions to the administered aqueous extract in the case of the extract treated animals. No death was recorded in these treated groups during the observation period.

The mean residual parasites recovered with time from the blood of Control and extract treated animals following initial loading of parasite density at D0 are summarized in Table 2. A diminishing trend of mean residual parasitemic density was observed especially in the treated mice as compared to the Control. The observed trend appeared dose dependent.

Table 2: Measured mean Residual parasite density with time

GROUP	TIME (day)							
	0	1	2	3	4	5	6	7
I	139657	95444	87543	87349	90192	90192	90192	90437
II	–	–	–	–	–	–	–	–
III	101927	73567	56843	55843	54732	53258	44924	35244
IV	101927	57321	38334	37432	24273	16678	15789	13567
V	175267	69447	41121	37986	36959	34959	20542	18324

The % Residual parasite density (%RPD) with time is shown in Table 3. The values were determined as mean residual parasite density (MRPD) at each time point as % of MRPD at initial time (DO).

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Table 3: % Residual Parasite Density with time

GROUP	TIME (day)							
	0	1	2	3	4	5	6	7
I	100±10	68.3±8	62.7±6	62.5±6	64.6±7	64.6±7	64.6±7	64.8±7
II	–	–	–	–	–	–	–	–
III	100±10	72.2±7	55.8±6	54.8±6	53.7±5	52.3±5	44.1±4	34.6±4
IV	100±10	56.2±6	37.6±4	36.7±4	23.8±2	16.4±2	15.5±2	13.3±1
V	100±10	39.6±4	23.5±2	21.7±2	21.1±2	20.0±2	11.7±1	10.4±8

In the control group % RPD with time was on the average 64.58±7% in course of the study. Extract treatment exerted a statistically significant effect. Administration of the extract significantly moderated % RPD with time in all extract treated mice compared with control group ($p < 0.05$). The observed moderating effect by the extract was significantly dose dependent, being lowest in the animals receiving higher extract doses ($p < 0.05$). The %RPD in animals receiving 200mg/kg extract was significantly lower than the Control but as well significantly higher than values of 400 and 800mg/kg treated animals ($p < 0.05$). No statistically significant differences ($p < 0.05$) were noted in %RPD in the animals treated with 400mg/kg vis-à-vis 800mg/kg (Table 3).

The overall antiplasmodial efficacy/efficiency of the extracts at various concentrations were determined at the end of the study on day 7, from table 2 using the relationship:

$$\frac{Control\ parasite\ density\ -\ Extract\ parasite\ density\ (of\ each\ dose)}{Control\ parasite\ density}\ x\ 100$$

The values obtained are presented as % plasmodial suppression at the end of the study. These values are summarized in Table 4. Extract significantly suppressed plasmodial parasitemia at all the extract doses considered in this study ($p < 0.05$).

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Table 4: Summary of time required to reduce parasite to 50% (Ct½) of initial density at time D0 and % plasmodial suppression at the end of study (7d)

Group	PARAMETER	
	% plasmodial suppression	Ct½ (d)
I	00.0	> 7.0
II	–	–
III	61.0±6	4.8±05
IV	85.0±9	2.3±0.3
V	80.0±7	1.4±0.2

The logarithm plot of transformed % residual mean parasite density (RMPD) against time in Control and extract treated animals is depicted in Figure 1.

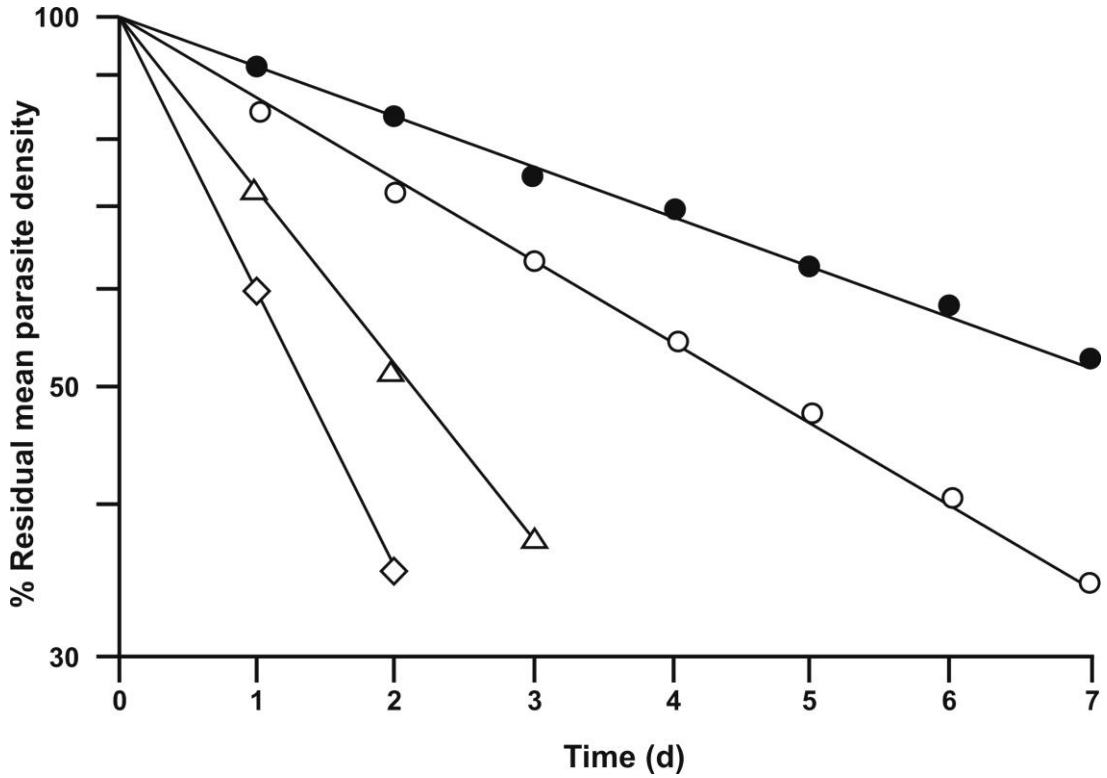

Figure 1: Residual mean parasite density (%) with time in Control (●) and groups treated with 200mg/kg (○); 400mg/kg (Δ) and 800mg/kg (◊) of aqueous P. niruri leaf extract

The clearance time (d) required to clear 50% of manifest parasitemia (Ct½) in the Control and treatment groups are also displayed in Table 4. Ct½ values were

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extrapolated from the logarithm plot of Residual mean parasite density (%) with time (Figure 1).

Ct½ was more than 7d in the Control. In the treated groups, Ct½ was significantly dose dependent ($p < 0.05$), and response appears to be additive. All the treated group exhibited Ct½ significantly lower than Control value ($p < 0.05$). The 200mg/kg had a Ct½ of 4.8d which is significantly longer than 400mg/kg and 800mm/kg ($p < 0.05$). On the other hand, Ct½ of 2.3d at 400mg/kg was significantly shorter than Ct½ at 200mg/kg but significantly longer than Ct½ of 1.4d in the 800mg/kg ($p < 0.05$). The overall order of demonstrated antiplasmodial potency based on their Ct½ was Control < 200mg/kg < 400mg/kg < 800mg/kg: > 7d > 4.8d > 2.3d > 1.4d respectively ($p < 0.05$) (Table 4).

The function test indicator enzymes for the liver, serum aspartate transferase (AST) and alanine transferase (ALT) of the control and parasitized mice treated with varying doses of aqueous extract of P. niruri leaves are summarized in table 5.

Table 5: Summary of liver function tests in Control and parasitized mice treated with different doses of aqueous extract of P. niruri leaves

GROUP	PARAMETER	
	AST (mg/dl)	**ALT (mg/dl)**
I	38.0±5	69.5±8
II	37.6±4	68.4±7
III	29.6±2	55.9±4
IV	31.9±3	56.3±5
V	35.1±4	67.2±7

Evident from Table 5, there were essentially no significant differences in the values of AST and ALT in the extract treated mice when compared with the Control or Sham mice ($p > 0.05$). Similar trend was observed in renal function assessed by measured serum concentrations CR ($p > 0.05$) in these animals. As well, changes in blood urea nitrogen (BUN) following the treatment of the mice with the various doses of the extract were not as statistically impressive relative to the Control or Sham (Table 6).

Table 6: Summary of renal function tests in Control and parasitized mice treated with different doses of aqueous extract of P. niruri leaves

GROUP	PARAMETER	
	Urea (mg/dl)	**Creatinine (mg/dl)**
I	90.0±9	2.8±0.3
II	89.0±9	2.4±0.2
III	63.4±6	1.5±0.2
IV	78.2±8	2.0±0.2
V	79.0±8	2.4±0.3

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The slight alterations observed in the blood concentrations of these variables failed to alter their normal blood levels of these variables, BUN and CR respectively, observed in the Sham animals (Tables 5 & 6).

DISCUSSION

The results of this study demonstrate a severe virulence of the rodent plasmodium berghei species and the pathologic susceptibility of the animal model, swiss albino mice employed in the study. This observation is in accord with published evidence in literature. The parasite rapidly causes fulminating infection in these laboratory rodents leading to death within 1 – 3 weeks, especially in the younger animals with symptoms as observed in this study (Sinden, 1997).

The various doses of aqueous leaf extract of P. niruri utilized in this study demonstrated a potent antiplasmodial activities against the established P. berghei infection in this study. The extract at daily doses of 200, 400, 800mg/kg were effective at suppressing the induced rodent malaria and the response was dose dependent. The median Ct½ was 2.25d. This observed therapeutic efficacy is consistent with evidence in literature. Ajala et al., (2011) demonstrated antiplasmodial effect of the extracts and formulated capsules of P. amarus on a resistant malaria parasite strain, P. yoelii induced malaria infection in swiss albino mice. These authors employed both aqueous and ethanolic extracts of whole plant at daily doses of 200mg/kg – 1600mg/kg in a prophylactic and therapeutic settings.

Similar to this present study, the authors demonstrated a dose-dependent prophylactic and therapeutic effects with aqueous preparation being more potent than the ethanolic extract. In accord with the results of the present study, their extracts significantly delayed dose dependently, the onset of infection with a 79% suppression at the dose of 1600mg/kg/d, a dose which is twice the highest dose employed in this present study. Perhaps the high dose employed by the authors may be explained by the resistant malaria parasite strain, P. yoelii employed by these authors. Additionally, there seems to be differential effectiveness and potency of application of extracts of P. niruri plants: the leaves being more potent than stem or the roots (Soh et al., 2009). Thus, variable results can be obtained depending on portions and form of extraction of P. niruri viz the aqueous leaf extract being more potent than ethanolic extract vis-à-vis the whole plant extract. In this present study, a superior result was obtained with purely aqueous leaf extract at lower application dosages. The current % plasmodial suppressions ranged from 61% observed in the lowest dose of 200mg/kg/d to more than 80% in the higher doses of 400 and 800mg/kg/d employed in this current study.

Finally, the results of this study have demonstrated the safety of the extract at studied doses. The animals did not show any untoward effects to the extracts. The normal functions of the liver and renal systems were not adversely impacted by the extract. These findings are in tandem with published literature evidence of safety of P. niruri

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extracts and its hepatoprotection (Bhattacharjee and Sil, 2006). This safety as evident in this study is corroborative to published instances of demonstrated safety where extracts of phyllantus family were generally administered even in more precarious physiologic settings (Ansari et al., 2017; Harish and Shivanandapppa, 2006). This high safety factor as observed in this study were not countered by investigators working with even higher doses (Ajala et al., 2011).

CONCLUSION

In concluding, the results of this study further confirm the high susceptibility of swiss albino mice to plasmodial parasitemia and hence by phylogeny, a very useful animal model for evaluating relatively the human malaria pathophysiology. The study has equally demonstrated potent antiplasmodial effectiveness of aqueous leaf extract of P. niruri at significantly lower dosage relative to published comparative studies in literature, with a demonstrated safety akin to the extract. Overall, the present work has shown, in an animal model of malaria, a high efficacy of extracts of P. niruri traditionally used in chemotherapy of P. falciparum infection in humans. So the naturopathic use of these plants to treat malaria is based on a real antiparasitic activity.

FUTURE RESEARCH

Efforts are currently underway to further define and extend the findings of this study to establishing the potential febrifugal effectiveness of P. niruri aqueous leaf extract as a febrifuge in other hyper-febrile states in view of the current global febrile stance, with the ultimate aim of proposing a plausible mechanism the action of P. niruri leaf extract.

CONFLICT OF INTERESTS

The authors hereby declare no conflict of interests

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