Anti-Plamodial Activity of ethanolic root bark extract of *Piliostigma thonningii* Schum. (Caesalpiniacea*)* in mice infected with *Plasmodium berghei berghei* NK 65.

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ABSTRACT: *Piliostigma thonningii* Schum. (Caesalpiniacea*)* is used traditionally in the management of fever, cough, wounds and various ulcerations. Oral acute toxicity of the ethanolic root bark extract of *Piliostigma thonningii* was evaluated in mice using modified Lorke's method. The ethanolic root bark extract was evaluated for *in vivo* anti plasmodial activity against chloroquine sensitive strain of *Plasmodium berghei berghei NK65* in mice. Four day suppressive test, curative effect against established infection and prophylactic models of anti plasmodial studies were carried out. The oral median lethal dose was determined to be ≥ 5000 mg/kg body weight. The extract (100, 200 and 400 mg/kg) exerted dose dependent chemo suppressive effects at the different levels of the infections tested. However the anti-plasmodial effect of chloroquine at 5mg/kg body weight was higher than the extract in all the test models. This shows that the plant has anti plasmodial property that can be explored for the management of malaria.

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INTRODUCTION

Malaria is an endemic infectious disease that is wide spread in tropical and subtropical regions of the world and one of the six most important parasitic diseases of man (WHO, 1998). It is a major public health problem in sub-Saharan Africa, where over 85-90% of all global burden of malaria exists with up to 50% of all out patient visits in areas with high malaria transmission and 30% - 50% of all hospital admissions are attributed to malaria (WHO, 2005). The disease kills 1.1 million people world wide each year. Approximately 1 million of these deaths are in Africa and an estimated 700,000 of them are children. These malaria deaths account for one out of every four childhood deaths in Africa (UNICEF, 2000).

Although an effective vaccine is the best long term control option for malaria, current research on vaccine development is still at preclinical stage, and considering the different phases of vaccine development it is predicted that a reliable malaria vaccine is several years away. The global strategy for malaria mainly focuses on case management through provision of drugs capable of reducing the morbidity and mortality of malaria through provision of drugs capable of reducing or eliminating parasites (WHO, 1993; Schapira, 1994).

However, multiple anti-malarial drugs - resistant *Plasmodium falciparum* and the emergence of insecticide resistant *Anopheles mosquitoes* is causing not only the spread of malaria to new areas but also its re -emergence in areas where it had previously been eradicated (Collins and Jeffery 1996). This has prompted research towards the discovery and development of new, safe and affordable anti- malarial chemotherapies.

In this respect, plant resources are potential targets for research and development of alternative malarial drugs, with novel modes of action (Muregi *et al.* 2003). Although up to 80% of the African population uses traditional medicine especially plant remedies for the management of diseases including malaria, plants are not yet fully explored (WHO, 2002b).

Piliostigma thonningii belongs to the family caesalpiniaceous and it is a shrubby tree with alternate compound leaves. The fruits are often pod-like with pods containing one to many seeds. The tree is perennial in nature and its petals are white to pinkish colour produced between November and April. While the fruits, are hairy, hard, flattish pod turns rusty brown, woody and twisted which splits at ripening and usually persistent on the tree are produced between June and September (Lock and Simpson, 1999). Locally, the seed is called Abafe in the Yoruba land (Nigeria). Other names include Monkey bread, Camel's foot, Kalgo (Hausa) and Okpoatu (Ibo). *P. thonningii* grows in open woodland and savannah regions that are moist and wooded grassland in low to medium altitudes. It is widely distributed in Africa and Asia. It is foundgrowing abundantly as a wild uncultivated tree in many parts of Nigeria such as Zaria, Bauchi, Ilorin,

Plateau, Lagos and Abeokuta (Schultes and Hofmann, 1973;Djuma, 2003). In Nigeria it is represented by two species that are much alike - *Piliostigma thonningii* and *Piliostigma reticulatum* (Keay *et al.* 1964). A warm infusion of the bark and leaves traditionally is used to relieve fever and toothache. The powdered bark or the young inner bark and the scurf scraped from the surface of the pods are applied as dressing for wounds. The bark is also chewed for the relief of cough. The leaves and bark are believed to have expectorant property, and are used in infusions or chewed for chest complaints, intestinal troubles, diarrhoea and dysentery (Dalziel, 1937).

This study was aimed at evaluating the anti - plasmodial activity of ethanolic root bark extract of *Piliostigma thonningii on Plasmodium berghei berghei infection* in mice.

MATERIALS AND METHODS

Collection and Identification of plant material:

Fresh root bark of *Piliostigma thonningii was* collected from Suleja, Niger State Nigeria. It was identified by Mal. Ibrahim Muazzam of the Medicinal Plant Research and Traditional Medicine (MPR & TM) Department, NIPRD Idu, Abuja. A sample with voucher number NIPRD/H/6268 has been deposited for future reference at the department's (MPR & TM) herbarium.

Extraction of plant material:

The root bark was air dried under shade. The dry root bark was ground to coarse powder in a mortar. Extraction was carried out by dispersing 500g of the ground plant material in 2.5 litres of 70% ethanol and shaking was done with GFL shaker (No 3017 MBH, Germany) for 72h. This was followed with vacuum filtration and extract concentration using a rotary evaporator at a temperature not exceeding 40^0 *C.* The concentrate was heated over a water bath to obtain a solvent free extract, which was stored in a refrigerator, at 4^0 C.

Animals:

4 weeks old albino mice weighing 18-22gobtained from the Animal Facility Centre of NIPRD Abuja were used for the study. They were housed in plastic cages with saw dust as beddings and given food and water *ad libitum.* The mice were used in accordance with NIH Guide for the care and use of laboratory animals; NIH Publication (No.83-23) revised (1985) NIPRD-Standard Operation Procedures (SOPs).

Phytochemical Screening:

The extract was screened for the presence of secondary metabolites and constituents using conventional protocols for detecting the presence of alkaloids, tannins, saponins, and resins (Trease and Evans, 1996).

Acute Toxicity Test (LD_{50})

Acute toxicity of *Piliostigma thonningii* ethanolic root bark extract was carried out using modified Lorke' s method(1983).The study was carried out in two phases . In phase one of the study nine mice were randomized into three groups of three mice each and were given 10,100and 1000 mg/kg body weight of the extract orally. The mice were observed for signs of toxicity which include but not limited to paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first four hours and subsequently daily for 7 days.

In the second phase of the study another fresh set of nine mice were randomized into three groups of three mice each and were given 1600, 2900 and 5000 mg /kg body weight of the extract orally based on the result of the first phase. These were observed for signs of toxicity and mortality for the first critical four hours and thereafter daily for 7 days. The LD_{50} was then calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose i.e. the geometric mean of the consecutive doses for which 0 and 100% survival rates were recorded in the second phase. The oral median lethal dose was calculated using the formula:

$LD_{50}= \sqrt{}$ minimum toxic dose × maximum tolerated dose

Rodent parasite (*Plasmodium berghei berghei);*

The rodent parasite *Plasmodium berghei berghei* NK 65 was sourced from National Institute for Medical Research (NIMR), Lagos, Nigeria and kept at Animal Facility Centre, NIPRD Idu, Abuja, Nigeria. The parasites were kept alive by continuous intraperitoneal passage in mice (Adzu *et al.* 2007) every four days. The re - infected mice were used for the study. Prior to the beginning of the study, one of the infected mice was kept and observed to reproduce disease symptoms similar to human infection (English *et al.* 1996).

ANTI - PLASMODIAL STUDIES

Suppressive Test:

The Peter's 4-day suppressive test against chloroquine sensitive *Plasmodium berghei berghei* NK 65 infection in mice was employed (Peters, 1965). Adult Swiss albino mice weighing 18-22g were inoculated by intraperitoneal (I.p) injection with standard inoculum of *Plasmodium berghei berghei* with 1 x 10^7 infected erythrocytes. The mice were randomly divided into 5 groups of 6 mice per group and treated for 4 consecutive days with 100, 200 and

400 mg extract/kg body weight orally daily respectively. Two control groups were used; the positive control was treated daily with 5mg chloroquine/kg while the negative control group was given 5ml/kg normal saline. On day 5 of the experiment, blood was collected from the tail of each mouse and smear on to a microscope slide to make a film (Saidu *et al*., 2000).The blood films were fixed with methanol, stained with10% Giemsa at pH 7.2 for 10 minutes and parasitaemia (WHO, 1994) examined microscopically. The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice.

Evaluation of Schizontocidal Activity of *P. thonningii* on established infection (Curative or Rane test):

Evaluation of the curative potential of *P. thonningii* root bark extract was carried out according to the method described by Ryley and Peters (1970). The mice were injected intraperitoneally with standard inoculum of 1 x 10^7 *P. berghei berghei* NK 65 infected erythrocytes on the first day (day 0). Seventy two hours later, the mice were divided into five groups of five mice each. The groups were orally treated with *P. thonningii* root bark extract (100, 200 and 400mg/kg/day), chloroquine (5mg/kg/day) was given to the positive control and an equal volume of distilled water was given to the negative control group. The treatment was carried out once daily for 5 days and blood smears were collected and examined microscopically to monitor the parasitaemia level. The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post inoculation) in each group over a period of 28 days (0-27).

Evaluation of Prophylactic Activity of *P. thonningii* (Repository test):

Evaluation of the prophylactic potential *P. thonningii root* bark extract was carried out according to the method of Peters (1965). Treatments were initiated on D0 and continued till D4 when the mice were all infected with the parasite. Blood smears were then made from each mouse 72 h after treatment (Abatan and Makinde, 1986) and increase or decrease in parasitaemia determined.

Statistical Analysis

The one way ANOVA test was used to analyze and compare the results at a 95% confidence level. Values of $P \leq 0.05$ were considered significant. Results were expressed as mean ± standard error of mean.

RESULTS

Phytochemical Screening

Results obtained from the phytochemical screening of the ethanolic root bark extract of *P. thonningii* showed the presence of carbohydrates, free reducing sugars, tanins, flavonoids, phenols, saponins, alkaloids, balsams, terpenes, steroids and volatile oils while resins and glycosides were absent as shown in table 1.

Table 1. Phytochemical Composition of Ethanolic Root Bark Extract of *P. thonningii.*

Phytochemicals (Constituents)	Inference
Carbohydrates	Present
Free reducing sugars	Present
Tanins	Present
Flavonoids	Present
Phenols	Present
Saponins	Present
Alkaloids	Present
Balsams	Present
Terpenes	Present
Steroids	Present
Volatile oils	Present
Resins	Absent
Glycosides	Absent

Acute Toxicity Test:

Behavioural signs of toxicity observed in mice given 1000mg extract/kg body weight and above include; paw licking, salivation, stretching and reduced activity. There was however no mortality at all the dose levels used. The median lethal dose LD_{50} was estimated to be ≥ 5000mg mg/kg body weight

Suppressive Effect:

The ethanolic extract root bark of *P. thonningii* exerted dose dependent chemosuppressive effect against *Plasmodium berghei berghei* malaria parasite. The extract caused a significant($P \leq 0.05$) chemo suppression of 48.24%, 64.32% and 89.30% respectively, when compared to the control. The standard drug, chloroquine caused chemo suppression of 92.90%, which was higher than those of the extract treated groups (Table 2).

Table 2. Suppressive Effect of *P. thonningii* ethanolic root bark extract and chloroquine against *P. berghei berghei* infection in mice.

Treatment	Parasite count	% Suppression
Normal saline 5ml/kg(control)	11.20± 9.26	-
Extract 100mg/kg	5.82 ± 3.66*	48.24
Extract 200mg/kg	4.00 ± 1.03*	64.32
Extract 400mg/kg	1.20 ± 1.26**	89.30
CQ 5mg/kg	0.80 ± 0.75**	92.90

*- significant at (P≤ 0.05) from the control, ** - Highly significant at (P≤ 0.01) from the control

Curative Effect

It was observed that the ethanolic root bark extract of *P. thonningii* produced daily, dose dependent reduction in parasitaemia levels in the extract treated groups, with a similar reduction in the chloroquine treated group (positive control). While there was a daily increase in parasitaemia in the negative control group. The average percentage suppression of parasitaemia of the extract treated groups on day 7 were 49.52%, 61.22% and 64.85% for the 100, 200 and 400mg/kg/day of the extract respectively. While that of the chloroquine treated group was 92.59% (Table 3).

Table 3. Curative Effect of *P. thonningii* ethanolic root bark extract and chloroquine against *P. berghei berghei* infection in mice.

Treatment	Parasite count	% Suppression
Normal saline 5ml/kg(control)	16.50 ± 6.84	-
Extract 100mg/kg	8.33 ± 5.33*	49.52
Extract 200mg/kg	6.40 ± 5.03*	61.22
Extract 400mg/kg	5.80 ± 1.37**	64.85
CQ 5mg/kg	1. 20 ± 0.96**	92.59

*- significant at P≤ 0.05 from the control, ** - Highly significant at P≤ 0.01 from the control

Prophylactic Effect:

The ethanolic root bark extracts of *P. thonningii* exerted significant (P≤ 0.05) dose dependent reduction in level of parasiteamia of 24.52 %, 43.40%, 56.60% and 81. 13% at 100mg/kg, 200mg/kg, 400mg/kg and the chloroquine treated groups respectively (Table 4).

Table 4. Prophylactic Effect of *P. thonningii* ethanolic root bark extract and chloroquine against *P. berghei berghei* infection in mice.

Treatment	Parasite count	% Suppression
Normal saline 5ml/kg(control)	10.65± 2.07	-
Extract 100mg/kg	8.00± 1.22*	24.52
Extract 200mg/kg	6.00± 0.71*	43.40
Extract 400mg/kg	4.60± 0.89**	56.60
CQ 5mg/kg	2.00± 0.71**	81.13

* Significantly different from the control at P≤ 0.05

**Highly significantly different from the control at P≤ 0.01

DISCUSSION

The rodent model of malaria has been employed in this study for prediction of efficacy of anti malarial effect of *Piliostigma thonningii* root bark extract. Several conventional anti malarial agents such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives have been identified using rodent malaria model (David *et al*., 2004). *Plasmodium berghei berghei* are used in the prediction of treatment outcomes, hence it was an appropriate parasite for the study. Since this parasite was sensitive to chloroquine, this drug was used as the standard drug in this study.

The choice of 4 weeks old mice for the study was done to avoid the effect of anaemia in the old mice and the effect of possible physiological changes associated with ageing may induce on the treatment outcome (Pierrot *et al*.,2003).The in vivo model was

employed for this study because it takes into account possible prodrug effect and possible involvement of immune system in eradication of infection (Waako *et al*., 2005). The oral median lethal dose of 3807.89mg/kg body weight obtained for the ethanolic root bark extract of *Piliostigma thoningii* is 19 times greater than the minimum effective dose of 200mg/kg. Earlier reports have shown that if the median lethal dose of a test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies. It was also reported that oral administration is about 100 times less toxic than the intraperitonial (Jutamaad.1998).The extract is therefore safe and this could explain the safe use of the plant by the local people who have been using it in traditional management of malaria in Nigeria. The ethanolic extract is therefore a good candidate for further studies.

The 4-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitaemia is the most reliable parameter. A mean group parasitaemia level of less than or equal to 90% of the mock-treated control animals usually indicate that the test material is active in standard screening studies (Peter and Anatoli.,1998). The results obtained from our study showed significant decrease in parasitaemia of Plasmodium *berghei berghei* infected mice treated with the ethanolic root bark extract of *Piliostigma thonningii*. This significant suppression of parasitaemia observed was dose dependent. The crude extract caused 81.36% suppression in parasitaemia of P berghei infected mice while chloroquine a standard antimalarial drug exerted 91.53% suppression. When a standard antimalarial drug is used I mice infected with *P. berghei,* it suppresses parasitaemia to non-detectable levels (Kiseko *et al*., 2000), this is in agreement with the effect of chloroquine in this study. The observed antimalarial activity is consistent with the traditional use of the plant as a herbal medication against the disease in Nigeria. The extract exerted significantly repository effect in mice treated with 200 and 400 mg/kg body weight respectively (table 2).This effect was however lower in group that received low dose. This effect may be due to short duration of action of the extract occasioned by rapid metabolism and so parasite clearance could not be total. It may also be explained by the fact that not all anti–malarials are completely active against *Plasmodium berghei* model (Dow *et al*., 1998).The extract demonstrated a significant prophylactic effect against p. berghei infected mice as demonstrated by the reduction in the level of parasitaemia dose dependently (table3).It is evident based on these findings that *P.thonningii* possess potent anti-plasmodial effect justifying its folkloric usage in the management of anti malarial. These effects may be attributed to the presence of alkaloids, terpenes and flavonoids that screening of plants has implicated in antiplasmodial activity (Phlipson and Wright, 1990; Christensen and Kharazmi, 2001). However, the active principle(s) are yet to be identified and there is need for the identification. In view of this fact, attempts are being made to carry out antiplasmodial guided fractionation of the ethanolic extract to isolate the active compounds and also to test for the cytotoxicity of the extract.

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