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Antiplasmodial activities of *Parkia biglobosa* leaves: *In vivo* and *In vitro* studies

Modupe Builders[1*], Noel Wannang[2] and John Aguiyi.[2]

[1]*Department of Pharmacology, College of Health sciences, Bingham University, Jos, Plateau, Nigeria*
[2]*Department of Pharmacology, University of Jos, Nigeria*

ABSTRACT

The wide spread resistance of malaria parasites to conventional anti-malarials have stimulated the search for new drug entities especially those with new modes of action. The efficacy of the preparations of Parkia biglobosa leaves for the treatment of malaria have been widely acclaimed by the Hausa communities of northern Nigeria, thus the need to authenticate this claims as a critical step in the search for a new anti-malarial. The antiplasmodial activity of P. biglobosa was evaluated in vivo and in vitro against Plasmodium berghei berghei and clinical isolates of Plasmodium falciparum respectively. In the in vivo study, effects of graded doses of the extract were evaluated in mice infected with P. berghei berghei, while in the in vitro test the inhibitory effects of graded concentrations of the extract (3.125 to 100 µg/ml) on P. falciparum was evaluated. The oral median lethal dose (LD_{50}) in mice and preliminary phytochemical screening were also evaluated. There was a dose dependent inhibition of parasitaemia in the in vivo antiplasmodial tests, with maximum effect at 600 mg/kg. The in vitro screening demonstrated a weak and concentration-dependent activity (56.23µg/ml > IC50 > 50µg/ml) of the extract against P. falciparum. The LD_{50} in mice was estimated to be greater than 5000 mg/kg and the phytochemical analysis revealed the presence of flavonoids, tannins, terpenes, saponins, cardiac glycosides, alkaloids and reducing sugars. The leaf extract of P. biglobosa contains biologically active principles that are relevant in the treatment of malaria, thus supporting further studies of its active components.

Keywords: *Parkia biglobosa, Plasmodium berghei berghei, Plasmodium falciparum,* antiplasmodial, malaria.

INTRODUCTION

Malaria is a protozoa disease, transmitted by the Anopheles species of mosquito carrying the *Plasmodium* parasite. Species of the genus *Plasmodium* including *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* are known to cause malarial infections in humans, virtually all deaths are caused by *P. falciparum* [1].

Despite the substantial progress made in the treatment of parasitic diseases, malaria remains a significant therapeutic challenge especially because of the wide spread resistance of malaria parasites to currently available anti-malarial agents, the resistance of the mosquito vectors to currently available insecticides, the limited success in the development of malarial vaccines and the debilitating adverse reactions of conventional anti-malarial drugs [2]. These have stimulated the search for new pharmacologically active agents that can overcome these barriers. There is a long standing tradition for the use of phytomedicines for the treatment of malaria. The plant kingdom remains a major target in the search of lead compounds and new drugs to treat this debilitating parasitic disease. Quinine isolated from *Cinchona* and quinghaosu from *Artemisia annua* L. for instance illustrates the potential value of herbal medicines for development of antimalarial drugs [3].

Parkia biglobosa (Jacq.) R.Br. ex G. Don (family Fabaceae) popularly known as the "African locust bean tree", it is a medium-sized tree growing up to 30 m in height. The leaves are alternate and bipinnately compound, the thick, fissured bark is dark-grey to brown in color, while the fruits when mature darken to a red-brown or brown and the hulls of the pods become hardened, smooth [4]. P.bi*globosa* have been used in the Nigeria and other West African rural communities to treat a variety of diseases [5-7].The efficacy of the various preparations of *P. biglobosa* are widely acclaimed by the Hausa communities of northern Nigeria for the treatment of malaria, diabetes mellitus and pains. The leaves are boiled and taken orally as a decoction as a treatment for malaria. Extracts from the Stem barks, fruits and seeds are also used to treat various ailments ranging from malaria, inflammatory diseases, and infections to diarrhea [5, 6, 8- 9]. Thus, medicines derived from *P. biglobosa* are especially of great value to the West African sub regional rural communities that cannot afford or do not have access to modern medicine.

The objective of this study is to authenticate the claimed antimalarial potential of the decoction of the leaves of *P. biglobosa* by assessing its antiplasmodial activities, *in vivo* against *P. berghei* infected Swiss albino mice and *in vitro* against clinical isolates of *P. falciparum* as a critical step in the development of a new antimalarial.

MATERIALS AND METHODS

2.1 Plant materials

The leaves of *P. biglobosa were* collected in the month of February, 2009 from Chaza village in Niger state, Nigeria. The plant was identified and authenticated by (Ethno botanist) Mallam Muazzam Wudil of the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria where a voucher specimen (NIPRD /H/6220) was deposited at NIPRD Herbarium for reference

2.1.1 Extraction of plant materials

The plant material was air dried under shade and samples were pulverized to a coarse powder. A 100 g quantity of the powder was boiled with 1 L of distilled water for 30 min. The decoction was decanted, centrifuged at 4500 rpm (Erweka, Germany) for 30 min and freeze dried. The total yield of the dark green extract was 10.49% w/w of crude starting material. The freeze dried powder was stored in an airtight container and used for the study.

2.2 Animals

Swiss albino mice (20 - 25 g) of either sex maintained at Animal Facility Centre (AFC) of the Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria were used for the study. The animals were fed ad *libitum* with standard feed (Ladokun feeds, Ibadan, Nigeria) and had free access to water. They were also maintained under standard conditions of humidity, temperature and 12 h light/ darkness cycle. The animals were acclimatized for two weeks before the commencement of the study. A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) regulations of the ENV [10]. The principle of laboratory animal care was also followed in this study [11].

2.3 Malaria parasites

The chloroquine sensitive *Plasmodium. berghei berghei* (NK-65) obtained from the National Institute for Medical Research (NIMR), Lagos, Nigeria and kept at the Department of Pharmacology and Toxicology, NIPRD, Idu, Abuja, Nigeria were used for this study. The parasites were kept alive by continuous re infestation (I.P) in mice every four days [12]. Parasitized erythrocytes were obtained from a donor- infected mouse by cardiac puncture in heparin and made up to 20 ml with normal saline. Animals were inoculated intraperitoneally with infected blood suspension (0.2 ml) containing 1x 10^7 parasitized erythrocytes on day zero. Infected mice with parasitaemia of 5-7% were allocated to eight groups of six mice each [13].

2.4. Chemicals

All chemicals were purchased from Sigma – Aldrich, USA.

2.5 Phytochemical tests

The phytochemical screening of aqueous extract of P. *biglobosa* leaves was carried out to determine the presence of the following compounds; alkaloid, flavonoids, tannins, anthraquinones, saponins, glycosides, sterols, resins, volatile oil, terpenes and phenols using standard procedures [14,15].

2.6 Acute toxicity test

The acute toxicity of the extract was determined using Lorkes method [16] with modifications. Briefly, the test was carried out in two phases. Phase 1: Nine mice were divided into three groups of three mice per group. The three groups were administered orally with graded doses (10, 100 and 1000 mg/kg respectively) of the extract. Phase 2: Another nine mice were divided into three groups of three mice per group, which received graded doses (1600, 2900 and 5000 mg /kg) of the extract respectively. The number of deaths in each group within 24 h was recorded and the final LD 50 values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) and the lowest lethal dose (where deaths occurred).

2.7 In Vivo Studies

2.7.1 Suppressive test

A total of fifty-four mice were used for this study. Each mouse was given standard intra - peritoneal inoculums of $1.0x10^7$ *P. berghei berghei* parasites with the aid of a 1 ml disposable syringe. The animals were divided into nine groups of six mice each. Different doses of the extract (50, 100, 200, 300, 400, 500 and 600 mg/kg/day) were administered orally to these groups. Chloroquine diphosphate 25 mg/kg/day was given as positive control and 0.2 ml of normal saline as negative control for four consecutive days (D_0 to D_3). On the fifth day (D_4), thick blood smears were prepared and blood films were fixed with methanol. The blood films were stained with Giemsa, and then microscopically examined with 100-x magnification. The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice [17].

2.7.2 Curative test

On the first day (D_0), standard inoculums of (1×10^7) *berghei berghei* infected erythrocytes were injected intraperitoneally. Seventy two hours later, the mice were randomly divided into nine groups of six mice each. Seven groups received graded doses of the extract (50, 100, 200, 300, 400, 500 and 600 mg/kg/day) for 5 days. The remaining two groups received Chloroquine diphosphate (25 mg/kg/day) and 0.2 ml of normal saline respectively for 5 days. Thick blood smears were prepared from tail of each mouse for 5 days, 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 (D_0–D_{27}) [18, 19].

2.7.3 Prophylactic test

Nine groups of six mice per group were used for the study. Seven groups were administered with 50, 100, 200, 300, 400, 500 and 600 mg/kg/day doses of the extract orally. The other two groups were administered with 25mg/kg/day of chloroquine diphosphate and 0.2 ml/mouse/day of normal saline orally. The animals were dosed for four consecutive days (D_0–D_3). On the fifth day (D_4), the mice were inoculated with *P. berghei berghei* infected red blood cells. Seventy-two hours later, the parasitaemia level was assessed by studying Giemsa stained blood smears [20, 21].

2.8 Antiplasmodial Assay

2.8.1Patients' selection

Three fresh blood specimens were collected from three patients suffering from fever and other malaria symptoms with confirmed infection by *P. falciparum.* Already prepared dried -in-acridine orange –stained thin smears were examined for *Plasmodium* species identification .The parasite density was determined by counting the number of infected erythrocytes among 20,000 erythrocytes. From each patient, 4ml of venous blood was collected in a tube coated with EDTA. Samples with monoinfection due to *Plasmodium falciparum* and a parasite density between 1 and 2% were used for the in vitro antimalarial tests [22].

***2.8.2 In vitro* test**

The assay was performed in duplicate in a 96-wellmicrotiter plate, according to WHO method *in vitro* micro test (Mark III) (WHO, 2001). RPMI 1640 (Sigma Company, USA) was the culture medium used for cultivation of *P. falciparum* [23, 24]. Dilutions were prepared from the crude

plant extract and the final concentrations prepared by dilution were (100, 50, 25, 12.50, 6.25, and 3.125 µg/ml). Negative controls were treated with solvent and positive controls (Chloroquine phosphate) were added to each set of experiments. 50 µl of blood mixture media was added to each well in plate and incubated in a candle jar (with gas environment of about 3% O2, 6% CO2and 91% N2) at 37.0^{0}C for 24–30 h [25, 26]. After incubation, contents of the wells were harvested and stained for 5 min in an already prepared dried -in-acridine orange reagent. The parasites were counted in five fields of vision (> 200 total cells) using a fluorescence microscope (Partec cyscope fluorescence microscope, Germany) at a magnification of 40.

2.9 Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM). The IC 50 values were determined graphically on a log dose-response curve (log concentration versus percent inhibition curves) by interpolation.

RESULTS

3.1 Phytochemical tests

The result of the phytochemical screening of the freeze dried aqueous extract of P. *biglobosa* is presented in Table 1. The analysis revealed the presence of saponins, tannins, flavonoids, terpenes, phenols, sterols, isoquinoline alkaloids, indole alkaloids, cardiac glycosides and reducing sugars. However resins, volatile oil and anthraquinones were absent.

Table 1: **Phytochemical composition of aqueous extract of *P. biglobosa* leaves**

Phyto –compounds	Remarks
Indole alkaloid	+
Isoquinoline alkaloid	+
Tannins	+
Saponins	+
Sterols	+
Terpenes	+
Glycosides	+
Phenols	+
Anthraquinones	_
Flavonoids	_
Reducing sugars	+
Resins	_
Volatile oil	_

+ = Present; - = Absent

3.2 Acute toxicity test

In the acute toxicity test, *P. biglobosa* up to the dose level of 5000mg/kg of body weight did not inflict any lethality or toxic symptoms on the Swiss albino mice. The absence of death at doses up to 5000 mg/kg of the extract show that the LD_{50} of the aqueous leaf extract of *P. biglobosa* is higher than 5000 mg/kg P.O.

3.3 *In vivo* antiplasmodial study

The aqueous extract of *P. biglobosa* showed dose dependent reduction of parasitaemia at the different doses employed. The extract at a dose of 600 mg/kg showed maximum inhibition at 55.6

± 0.9%, 54.5 ± 1.6% and 51.6 ± 2.5% of parasitaemia for suppressive, curative and prophylactic tests respectively (Fig.1). The dose dependent chemosuppressive activities exhibited by the extract are similar to chloroquine (Fig.2). There was a dose dependent mean survival time in the mice treated with graded doses of the extract that ranged from 18.9 ± 2.0 - 28.0 ± 1.3 days , while the chloroquine diphosphate treated mice gave a mean survive time of 28.0± 1.1 (Fig. 3).

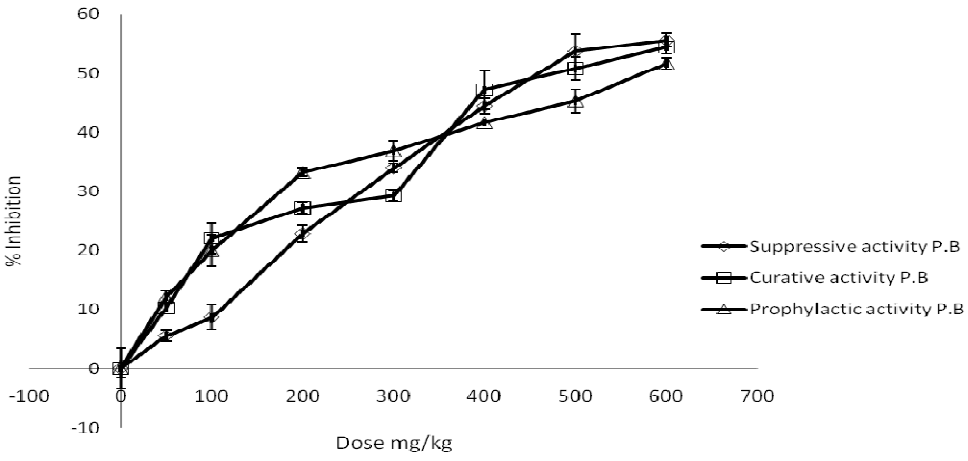


Fig.1: In vivo antiplasmodial activity of aqueous extract *P. biglobosa* leaves

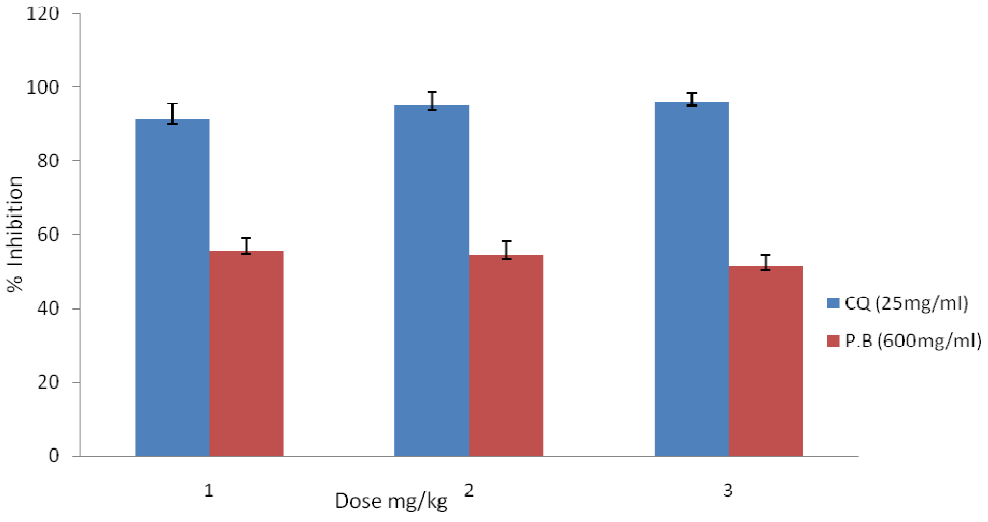


Fig. 2: In vivo antiplasmodial activity of aqueous extract of *P.biglobosa* leaves on P. *berghei*

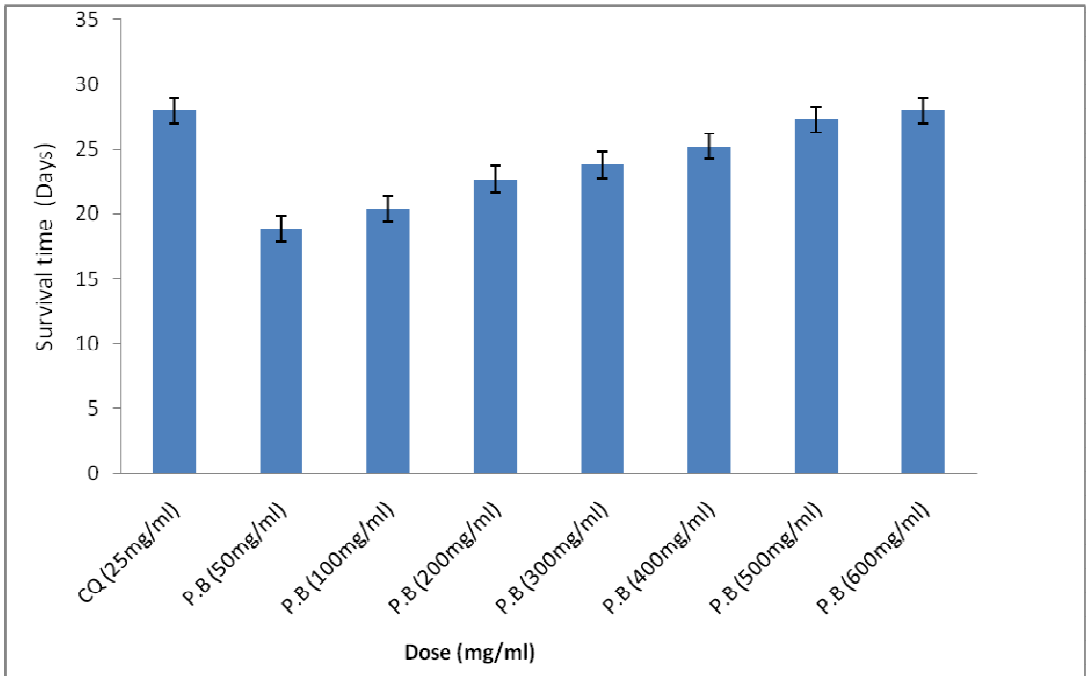


Fig.3: Mean survival time of mice treated with aqueous extract of *P. biglobosa* leaves versus chloroquine

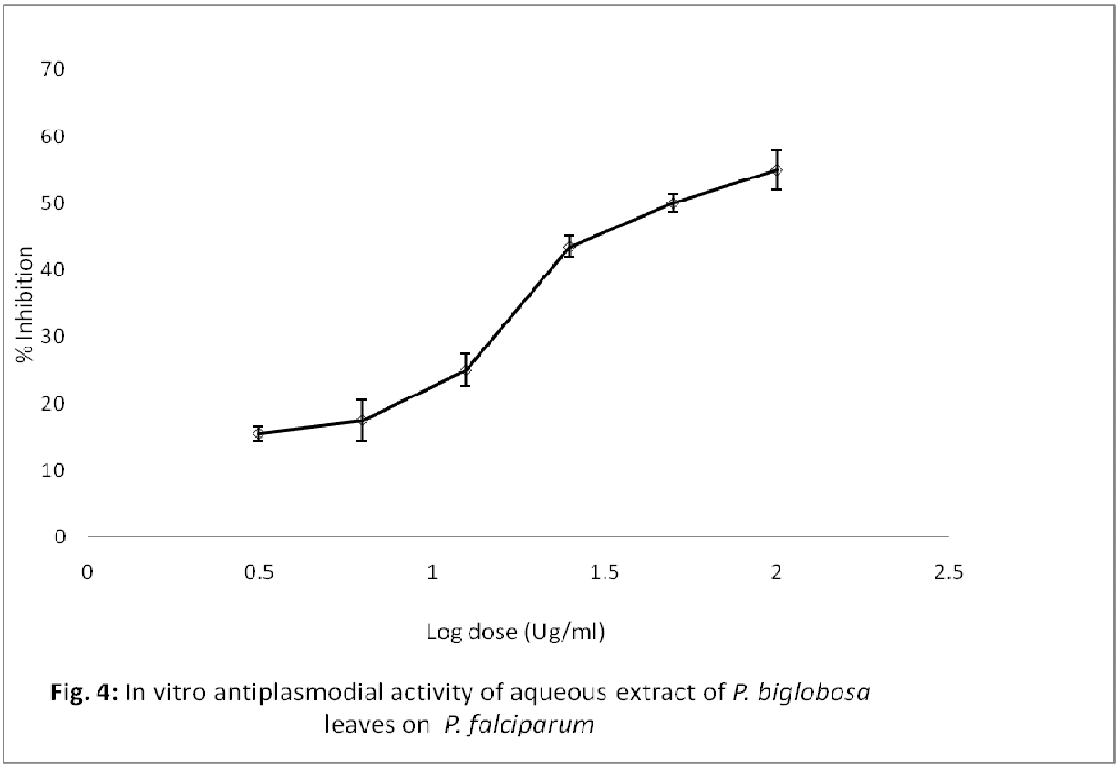


Fig. 4: In vitro antiplasmodial activity of aqueous extract of *P. biglobosa* leaves on *P. falciparum*

3.4 *In vitro* antiplasmodial study

The photomicrographs of the *in vitro* antiplasmodial activity of the extract are presented in Fig. 4. The extract showed a concentration dependent inhibition of *P. falciparum.* A maximum plasmodia growth inhibition of 55.0 ±2.0 % at a concentration of 100 µg/ml and 100 ±1.0% at a concentration of 0.2 ug/ml were obtained for the aqueous extract of *P. biglobosa* and chloroquine phosphate respectively (Fig. 5). The IC_{50} of the extract was 56.23 µg/ml (Fig. 6).

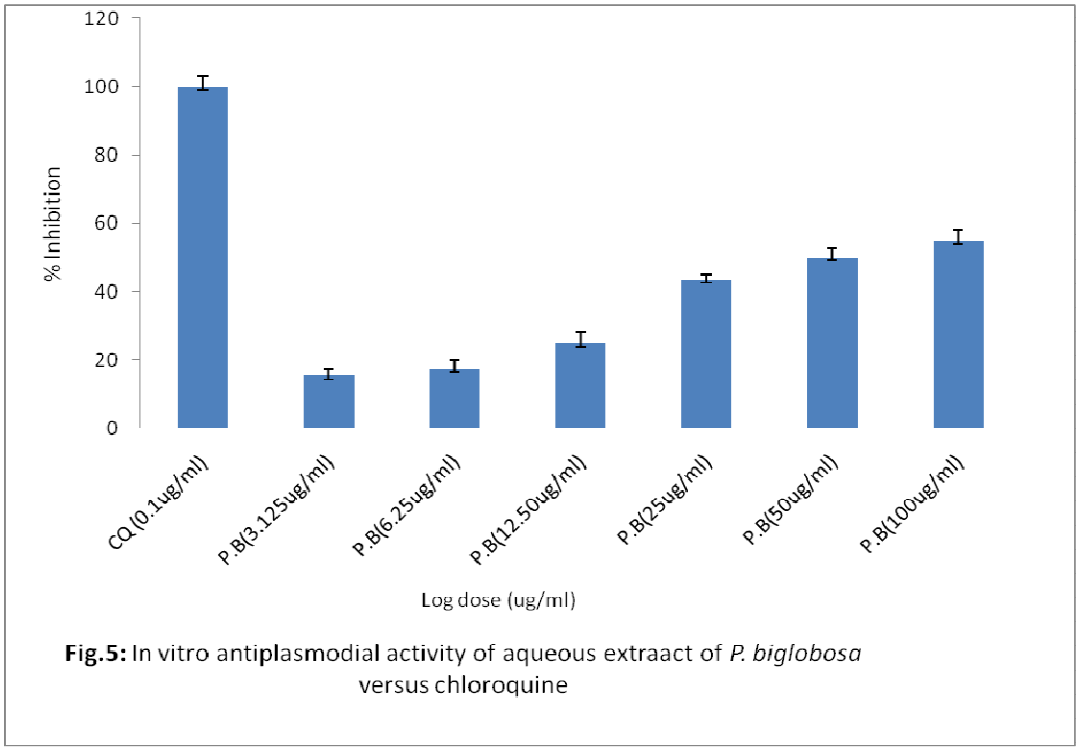
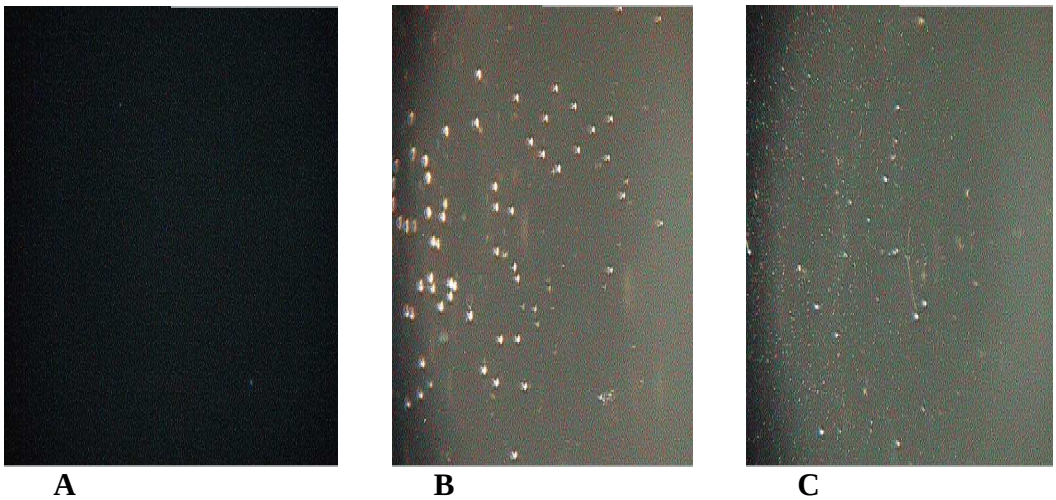


Fig.5: In vitro antiplasmodial activity of aqueous extraact of *P. biglobosa* versus chloroquine



A **B** **C**

Fig.6: Photomicrographs of In vitro antiplasmodial activity of the aqueous extract of *P. biglobosa*

***A**=Complete RPM1 medium; **B**=Untreated RPM1 medium with P. falciparium; **C**=RPM1 medium treated with P. biglobosa extract*

DISCUSSION

The *in vivo* and *in vitro* antiplasmodial activities of the extract show that the leaves of *P. biglobosa* contain biologically active substances that are relevant in the treatment of malaria. The leaves were prepared as decoction in order to mimic the folkloric method of preparation by the traditional medicinal practitioners. [5-6, 27-28]. The freeze-dried decoction was used so as to ensure the apparent stability and activity of the extract [13]. The oral dosing of the mice with the freeze dried extract was used as a replicate of the ethnomedical route of administration [29].

The acute toxicity of P .*biglobosa* has been investigated to determine any adverse effect that may arise as a result of a short time animal exposure to the aqueous extract within 24 h period. Though P .*biglobosa has* been used by TMPs without report of any mortality due to toxicity, this claim has been authenticated by the lack of death at oral treatment of over 5000 mg/kg body weight of the extract. The results thus suggest that the freeze dried decoction of the leaves of P *biglobosa is* acutely non toxic [30].

Chloroquine phosphate has been used as the standard antimalarial in this study for curative, suppressive and prophylactic antiplasmodial assessment because of its established activity on *P. berghei berghei* [31]. *P. berghei berghei* a rodent malaria parasite though, not able to infect man and other primates has been used because of its sensitivity to chloroquine [21, 32, 33]. A 4 day suppressive test was used to assess the efficacy of the extract by comparing the reduction in blood parasitemia and mouse survival time in the treated and untreated mice [34].The *P. biglobosa* extract showed similar antiplasmodial activities to chloroquine. The extract also showed a dose dependent chemosuppressive and curative activities against *P. berghei berghei* infected mice and also enhanced the mean survival time of the treated mice particularly the group administered with the 600 mg/kg/day of the extract.

The Micro-test (Mark III) is an established *in vitro* antiplasmodial assessment method that provides information on the quantitative drug response of *P. falciparum* irrespective of the patient's immune system was employed. This *in vitro* test permits the direct assessment of the extract on *P. falciparum* the causative agent of human malaria [22, 35].

The Cyscope fluorescence microscope is based on the use of Plasmodium nucleic acid-specific fluorescent dyes to facilitate detection of the parasites. Some of its attributes includes detection of parasites at low levels of parasitaemia due to its high contrast with the background, rapidity, sensitivity, reproducibility and ease of result interpretation [36]. The efficient contrast of the parasites and rapidity of this method is due especially to the use fluorescent dye, Acridine Orange [37].

The IC_{50} of the crude extract of *P. biglobosa* as determined was 56.23 µg/ml suggesting a weak antplasmodial activity relative to chloroquine phosphate. According to the norm the extract is active when IC_{50} <5 µg/ml, moderately active when 5 µg/ml < IC_{50} <50 µg/ml and weak when IC_{50} >50 µg/ml [38]. Though, the IC_{50} of *P. biglobosa* indicates a rather weak antiplasmodial activity in comparison to chloroquine phosphate, this value is however similar to those obtained for other crude extracts with established antimalarial activity [24, 39]. The higher values of IC_{50} obtained for the extract may be due to the crude nature of the extract. Plant crude extract are

often characterized by a variety of bioactive component with often diverse pharmacological activity. The isolation and purification of the target bioactive component usually results in boosted activity and low IC_{50}. The decrease in parasitaemia with increasing concentration of the extract also reflects an inhibitory activity on parasite replication thus supporting the isolation and development of the antibacterial agent.

The preliminary phytochemical screening of the freeze dried decoction of the aqueous extract of *P. biglobosa* revealed the presence of saponins, flavonoids, tannins, terpenes, isoquinoline alkaloids, indole alkaloids, cardiac glycosides, phenols, reducing sugars and sterols.

The therapeutic benefits of traditional remedies are often attributed to the presence of bioactive constituents present in the crude material [40, 41]. Many secondary metabolites of plants' origin have been shown to have antiplasmodial activity [42]. A number of naturally occurring alkaloids such as oxyacanthine, alstonerine, and cryptolepine isolated from *Dehaasia incrassate, Alstonia angustifolia* and *Crytolepsis sanguinolenta* respectively, had been reported to possess antiplasmodial activity [43- 45]. Triterpenoids such as meldenin, from Azadirachta indica, deacytlkhivorin from Khaya grandifolia and exiguaflavone from Artemisia indica have established antiplasmodial activities [46-48]. Similarly, a number of metabolites from plants such as saponins, flavonoids, tannins, cardiac glycosides, steroids etc., are reported to be responsible for the antiplasmodial activity of many medicinal plants [48-52]. It is therefore, probable that some metabolites present in *P. biglobosa* are responsible for its antiplasmodial activity.

Secondary metabolites are known effect antiplasmodial activities by a number of mechanisms such as elevating red blood cell oxidation and inhibiting the parasite's protein synthesis [19, 53]. Studies have shown that the leaves of *P. biglobosa* possess antioxidant activities which has been related to the presence of flavonoids and other phenolic compounds [54 - 56], these are known to counteract the oxidative damage induced by the malaria parasites [13, 57-58]. While it might be suggestive that this could be one of the mechanisms by which the aqueous leaf extract of *P. biglobosa* exert its antiplasmodial activity, it not possible to make such conclusion from this study as alkaloids and terpenes which are also known to exert effective antiplasmodial activity. This issue is pursued in future studies.

CONCLUSION

Crude extracts are the simplest available medications and are currently promoted by WHO as an alternative system of medication reaching a larger population especially in rural communities. The antiplasmodial activities of the freeze dried decoction of *P. biglobosa* collaborates the reason for the use of the aqueous preparations by the traditional medical practitioner in Northern Nigeria for the treatment of malaria. The antiplasmodial activity of this extract could be related to presence of certain biochemical agents with antiplasmodial potential. This study thus provides for the first time, the rationale for its application in traditional medicine for the treatment of malaria. Further studies are in progress in our laboratory to isolate and characterize the relevant bioactive components in the decoction of the leaves of *P. biglobosa*.

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