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Full Length Research Paper

Preliminary studies on *Piliostigma thonningii* Schum leaf extract: Phytochemical screening and *in vitro* antimalarial activity

A. Kwaji[1], P. U. Bassi[2], M. Aoill[1], C. M. Nneji[3] and G. Ademowo[3]

[1]Chemistry Department, Faculty of Science, University of Maiduguri, Borno State, Nigeria.
[2]Clinical Pharmacology Unit, Department Of Medicine, University of Maiduguri Teaching Hospital, Borno State, Nigeria.
[3]Institute for Medical Research and Training, College of Medicine, UCH, Ibadan, Oyo State, Nigeria.

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Different parts of *Piliostigma thonningii* Scum (Caesalpinioideae) have been used medicinally. The roots and twigs have been used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections in Eastern Nigeria. The leaf extracts has been used for various ethnomedicinal purposes including the treatment of malaria all over Eastern Nigeria. In this study, we have investigated the inhibitory effects of the crude ethanol and methanol extracts for *in vitro* antimalarial activity against chloroquine resistant *Plamodium Falciparum* clone (W2-Indo-China isolates). The aqueous screening using reported methodologies for phytochemical screening and i*n vitro* test methods, revealed the presence of alkaloids, tannins, saponnins, steroid, terponoids, flavonoids, cardiac glycosides and anthroquinoes. The crude leaf extracts obtained were tested for *in vitro* antimalarial activity using chloroquine resistant strain of *P. Falciparum* clone (W2 – Indochina isolates) . The 50% inhibitory concentrations (IC_{50}) were evaluated after 48 - 72 h contacts between the extracts and the parasite culture. The 50% inhibitory concentration values for both the crude amide ethanolic extract and the partially purified methanolic extract ranged between 6.20 - 15.06 µg/ml. While that of chloroquine was 0.316 µg/ml. This study suggested that *P. thonningii* leaf extract possess a significant level of antimalarial activity.

Key words: *Pilostigma thonningii* leaf, phytochemical screening, antimalarial activity.

INTRODUCTION

Malaria, though an ancient disease, is still Africa's leading health problem. It has a very high mortality and morbidity and is seriously crippling productivity (WHO, 2008). Each year, malaria affects about 300 - 400 million people worldwide, with at least 1 million deaths (Bryce et al., 2005; Hopkins et al., 2007; WHO, 2008). The major problem of malaria therapy in the tropics is attributed directly to worsening antimalaria drug resistance and poverty (Welcome News, 2002; Ekanem, 1997; White, 1999) . It therefore follows that if affordable and effective drugs are not available, morbidity and mortality due to malaria will continue to rise.

The world has relied largely since the 1950s, on chloroquine (CQ) a safe, inexpensive, widely available and once highly effective treatment for malaria. Unfortunately, resistance to chloroquine now occurs throughout the whole world. The problem of resistance seems to have been largely addressed by use of combination products such as artemisinin derivatives. (WHO, 2001; FMH, 2004,) Although the use of these combinations has resulted in more effective treatment of malaria, it has major setbacks of affordability and safety concerns. The rationale for this study therefore is to validate the claims of the safety efficacy of *P*. *thonningii* with the view to finding a safe and affordable antimalaria drug.

P. thonningii Schum is a leguminous plant that belongs to the Caesalpinioideae family which consists of about 133 genera (Hutchinson, 1958). The plant is perennial in nature and its flowers which are produced around November and December are white or pink in colour. It bears hairy flat-pod fruits that turn nesty-brown and

*Corresponding author. E-mail: pubassi@gmail.com

woody on maturity and usually persist on the plant till between June and September (Jimoh, 2005). Locally, the plant is known as Kargo (Hausa) monkey bread or camel's foot, inpilataki (Higgi) and kalur (Kanuri). *P. thorningii* is widely distributed in Africa and Asia (Akinniyi, 1983).

Different parts of *P. thonningii* have been used medicinally. For example, the roots and twigs have been used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections (Iwalewa et al., 1990; Jimoh, 2005). The leaf is useful in the treatment of malaria fever (Akinniyi, 1983; Rabo, 2001). In the absence of any scientific report, we designed an *in vitro* experiment to evaluate the antimalarial activity of alcohol extracts of the leaves of *P. thoninngii.*

MATERIALS AND METHODS

P. thoningii leaves were obtained from Kaltingo, Gombe State, North Eastern Nigeria in September 2004. The plant was identified by Dr E. T. Rabo, a botanist, in Biological Sciences Department, University of Maiduguri and a voucher specimen was preserved at the university of Maiduguri herbarium.

The sample was air-dried under shade at room temperature, adulterant were picked out of the leaves. The leaves were thereafter pulverized and 250 g was extracted three times for two hours with one litre of hexane to remove its lipophilic constituents as much as possible using a soxhlet extractor. The powdered leaves were again extracted using the same soxhlet extractor with ethanol/water system in the ratio of 8:2. These forms the crude plant extracts. Portions of these extracts were treated with hexane to remove all fat-soluble components, gums, resins and chlorophyll until there is a faint colour change in solvent of extraction. The extract was concentrated over a water bath and dried at 40°C to yield 64 g of the extracts. The ethanol extract was again dissolved in 80% methanol and eluted through a silica gel 60 g to afford a column fraction. The eluent was concentrated over a water bath and dried in a hot air oven at 40°C.

Crude drug preparations

About 0.01 g of the crude extract was carefully weighed, dissolved in 70 µl of dimethyl sulfoxide (DMSO) and finally made up to 10 ml with 70% ethanol. Crude extract is now 1 mg/ml, that is, 10 mg dissolved in 10 ml of solvent for each plant extract. These were kept in sterile falcon containers of 15 ml capacity each. The solutions were sonicated for 1 h to aid complete dissolution and kept refrigerated at 20°C until test. These now constitute the stock solutions. Both methanol and ethanol extracts were stored in refrigerator until required for the antimalarial test.

Preparation of 6% RBC

4.4 ml of culture medium with plasma was added to 0.6 ml of 50% RBC and then made up to 10 ml with complete culture medium (CMP) in a petri dish. Preparations of working solutions are:

1. Crude plant extracts: 2.5 ml of 1 mg/ml plant extract solution was added to 2.5 ml of culture medium (RPMI 1640 + HEPES + $NaHCO_3$). This gives working solution of concentration 500,000 ng/ml.

2. Standard drug chloroquine: About 10 mg of chloroquine was dissolved in 7 ml of absolute ethanol and 3 ml of distilled water was added, that is, 1 mg/ml. This constitutes the stock solution.

Working solution was made by taking 25 µl of drug and adding 4.975 ml of culture medium without plasma, such a dilution automatically gives q working solution of concentration of 5 µg (or 5000 ng/ml).

Phytochemical screening

The qualitative determination of the phytochemical constituents of *P. thonningii* leaves was carried out in accordance with the procedures outlined by Sofowora (1993), Falodun et al. (2005) and Trease and Evans (1978).

In vitro culture and estimation of *P. Falciparum* growth inhibition

The *P. falciparum* clone, W2- Indochina isolate, was cultured in plastic petri-dishes according to the methods of Trager and Jansen (1976), *in vitro* in human red blood cells (A+), diluted to 1% hematocrit in PRMI 1640 medium (Gibco-brl, Paisley, Scotland) supplemented with 25 mM HEPES, 30 mM $NaHCO_3$ and 10% human serum.

Drug testing was performed in duplicate in 96 well culture plates as described by Trager and Polanski (1981) with cultures containing mostly ring stages (time between liberation and re-invasion = 16 h) at 1% parasitememia (hematocrit = 1%). 100 µl of parasitized culture (hematocrit = 2%) and 100 µl of medium containing 50 ml of diluted crude extract were mixed in each well.

Dried extracts of plants were dissolved or micronized in ethanol and a series of 6 concentrations prepared by 10 fold dilutions in RPMI 1640 medium. The ethanol concentration for tested dilutions was not more than 0.1%. Aliquots of diluted crude drugs (50 ml) were dispensed into 96 well microtiter plates. All tests were performed in duplicates. Dilutions to produce 1% parasitaemia were made with uninfected washed red blood cells. Two series of controls were performed, one with parasitized blood without the addition of plant extracts and the second with infected (parasitized) blood in the presence of standard antimalarial agent chloroquine. The plates were incubated using candle jar at 37°C for a period of 48 - 72 h at which the culture was terminated. Thin smears were made from settled red cells. These were fixed in methanol, stained with 10% Giemsa stain and the parasitaemia level determined by visual microscopy.

Statistical analysis

These data were computer analyzed using the graph pad prism software (UK). Values are presented in tables as means ± SD, while percentage inhibition concentration of parasitaemia in extract was calculated using t-test, and P = 0.05 was considered significant.

RESULTS

The phytochemical screening shown in (Table 1) revealed the presence of alkaloids, tannins, saponins, steroid, terponoids, flavonoids, cardiac glycosides and anthraquinones. The inhibition rates of parasite growth by the two extracts of *P. thonningii* and chloroquine are shown in Tables 2 and 3, in order to obtain the 50% inhibitory concentrations of crude aqueous ethanol

Table 1. Preliminary phytochemical constituents of *P. thonningii* Schum leaf.

Chemical constituents	*P. thonningii*
Alkaloids	+
Tannins	+
Saponins	+
Steroids	+
Phlobatannins	-
Terpenoids	+
Flavonoids	+
Cardiac glycosides	+
Anthraquinones	+

+ = Present; - = Absent.

Table 2. The percentage inhibition of parasite growth at various concentrations of aqueous- ethanolic and methanolic crude extracts of *P. thongginii* Schum leaf.

	P_1 IC_{50}= 15.06 µg/ml		P_2= IC_{50}=6.20µg/ml		P_1 and P_2 Concentration
Wells	% of Parasitaemia relative to Control well	% Parasitaemia inhibition	% of Parasitaemia relative to Control well	% Parasitaemia inhibition	Extract concentrations (ng/ml)
A	100.00	00.00	100.00	00.00	00.00
B	61.50	38.50	4.30	95.70	50,000.00
C	73.00	27.00	34-80	65.20	16,666.67
D	53.90	46.00	46.70	53.30	5,555.56
E	65.40	34.60	67.50	32.80	1851.85
F	69.20	30.80	65.20	34.80	617.28
G	57.70	42.30	91.30	8.70	205.76
H	88.50	11.50	95.60	4.40	68.59

P_1 = Aqueous ethanol extract of *P. thonningii* Schum leaf.
P_2 = Aqueous methanol column chromatography fraction of *P. thonningii* Schum leaf extract.
IC_{50} = 50% inhibitory concentration.
B - H = 8 rows of culture plate wells consisting of 12 vertical columns.
A = control well.

extract of the plant, it lies within the range 6.20 - 15.06 ug/ml, close to that of azadirachta indica, IC_{50} = 4.17 – 7.29 µg/ml, which is commonly used to treat malaria. Chloroquine, IC_{50}= 0.316 µg/ml. In the Tables 2 and 3, 13-fold serial dilutions were made in wells A-G from stock solutions, while well, H, contains no plant extract or chloroquine and served as the control.

Mean value for the percentage inhibition concentration of parasitaemia of *P. thonningii* extract was calculated using t- test, for the six–fold dilution of crude and partially purified extracts. Between group P1 and P2, the results are statistically significant (P = 0.05).

DISCUSSION

The presence of phytochemical substances (especially the alkaloids and the flavonoids) in the leaf of *P. thonningii* may suggest pharmacological activity. This is, in keeping with antiplasmodial activity of these substances as reported by other workers like Kutchan, (1996), Iwalewa (1990), Raynes (1999), Picards (1996). Alkaloids were the first active constituents of medicinal plants used in the treatment of malaria in the western world (that came with the discovery of the medicinal properties of quinine, an alkaloid found in the bark of the cinchona tree (Rayness, 1999). Several other phytochemical substances such as tannins, flavoroids and phenolic compounds also have medicinal value (Edeoga, 2005). Plants therefore represent a useful source of antimalarial agents (Bernoit, 1996; Francoise, 1999).

The use of the initial information that the antimalarial activity of crude plant extracts could be obtained using chloroqinine- resistant strain of *P. falciparum* come from the works of O`Neill et al. (1985) which demonstrated for example, the IC_{50} of the crude ethanol extract of *Artemisia annua* for chloroquine- resistant strain K-I was found to be 3.9 µg/ml. Francoise et al. (1999) evaluated the *in vitro* antimalarial activity of aqueous leaf extract of *C. tinctorium* and found it to be 3.8 - 7.5 µg/ml. This

Table 3. Percentage inhibition of positive growth at various concentration of chloroquine as a standard antimalaria agent with IC_{50} of 0.316 ug/ml. The control week, H is considered as 100%.

Wells	% of parasitaemia relative to control well	% parasitaemia inhibition	Drugs concentration in ng/ml
A	0.00	100.00	500.00
B	38.00	62.00	166.67
C	84.00	16.00	55.56
D	88.00	12.00	18.52
E	92.00	8.00	6.15
F	96.00	4.00	2.08
G	100.00	00.00	0.68
H	100.00	00.00	0.00

IC_{50} = 50% Inhibitory concentration.
A – G = Culture plates.
H = Control well (the control well, H is considered as 100 %).
% (percentage parasitaemia) = the number of parasitized cells per 1000 red blood cells x 100.

Table 4. Statistical table.

Extract samples	Crude extract	Partially purified extract
	P1	P2
P. thonningii	34.07 ± 4.60^a P =0.000	42.03 ± 12.09^b P =0.013

informed the use of the clone, W2- *P. falciparum* Indochina isolate in this study, aqueous ethanolic and methonolic extract of *P. thonningii* in which the 50% inhibitory concentration (IC_{50}) was found to lie within the significant level of activity was observed in the two crude extract of *P. thonningii*) values of PI = 15.06 and P2 6.20 ug/ml, in addition, these values are also close to that of Neem tree (*Azadirachta indica*), IC_{50} = 4.17 and 7.29 µg/ml, which is used commonly to treat malaria in the tropics. There was an increase in activity for the column fractions, P2, relative to the crude ethanalic extract, P2, the IC_{50} values of 50 ug/ml for crude plant extract above which no activity exists.

Another finding observed in this study is significant difference in the levels of inhibition between two samples of *P. thonningii* extracts (P = <0.05) (Table 4). This means that the separation process was effective, since all other parameters such as initial parasitaemia inoculums size, drug concentration, total volume in each well and the incubation period for each row on the test palate was the same. Hence the variation in activity between crude and partially purified extracts of *P. thonningii* can be attributed to the effect of the separation process which is in line with other studies in literatures.

These high activities might help in dealing with the problem of Chloroquine- resistant malaria. This may therefore give credence to the use of *P. thonningii* extract in traditional medicine practice as an antimalarial agent.

Although toxicology study was not performed, extensive use of this plant by African native healers has not reported any adverse effects and this may suggests that no highly toxic components are present. It also implies that, since plant like *Azadirachta indica* was used universally in the management of malaria, it could be a potential source of chemotherapeutics agents, and giving the high inhibitory activity demonstrated in this study, the plant might be of help in tackling the problem drug resistant malaria.

In conclusion, this study has demonstrated that *P. thonningii* Schum leaf extracts possess antimalarial properties. This lends scientific credence to the use of the leaves for the treatment of malaria in ethnomedicine.

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