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Antiplasmodial Activity of the Ethanolic Extract and Flavonoid Fraction of the Stem of *Phyllanthus amarus* in Experimental Mice

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ABSTRACT: Malaria infestation in Nigeria is still worrisome, hence, the need for new antimalarial agents. This study, therefore, investigates the antiplasmodial activity of the ethanolic extract and flavonoid fraction of the stem of *P. amarus* in the blood, brain, liver and pancreas of *Plasmodium berghei* (chloroquine sensitive NK65 strain) infested mice. Forty (40) adult male BALB/c mice, weighing between 20-30g were randomly divided into eight (8) groups (n=5 mice per group) and treated with varying doses (150, 300 and 450 mg/kg/d) of either the ethanolic extract or the flavonoid fraction of the stem of *P. amarus* for seven (7) days. The animals were euthanized on the 8th day after an overnight fast under chloroform anaesthesia, and smears of the blood and tissues were processed for parasite count using Giemsa stain. Results show significant ($p< 0.05$) *in vivo* antiplasmodial activity by the ethanolic extract in a dose-dependent manner, which compared well with the standard chloroquine treatment. However, treatment with the flavonoid fraction yielded minimal growth suppression. The observed active antiplasmodial activity of the ethanolic extract of the stem of *P. amarus* is therefore, not traceable to the flavonoid phytochemical. The active antiplasmodial phytochemical(s) in the ethanolic extract of the stem of *P. amarus* need to be identified by further study.

Keywords: Malaria, Infestation, Antiplasmodial activity, *Phyllanthus amarus, Plasmodium berghei,* Flavonoid

Introduction

Malaria is a deadly disease caused by protozoans of the genus *Plamodium* that infest humans and several other animals. Malaria infestation occurs following bite from an infested female *Anopheles* mosquito (Biswajit, 2013). The known species of *Plasmodium* that infest humans include *Plasmodium falciparium, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and Plasmodium knowlesi*, with *Plasmodium falciparium* being the commonest and most severe among human population (Sutherland *et al*., 2010).
Malaria is particularly endemic in sub-Saharan Africa, followed by Asia, and the Americas (Chinwuba *et al*., 2015). In 2017, Africa was home to 92% of malaria cases and high rate of associated deaths. The following African countries accounting for nearly half (45%) of all malaria cases worldwide, Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%) and Uganda (4%) (WHO, 2019).
Despite actions to prevent and eliminate malaria, (such as sleeping under insecticide-treated net [ITN], indoor spraying with insecticides, use of antimalarial medicines, and trial vaccine, RTS,S/AS01[RTS,S], which has been

introduced in selected areas in Ghana, Kenya and Malawi), the estimated number of malaria deaths remained virtually unchanged (WHO, 2019). The need for the development of more potent antimalarial agents to combat the threatening scourge has, therefore, become urgent.

One of the chief sources of drug discovery is herbal plants. Many medicinal plants are traditionally used in Nigeria for the treatment of malaria (Okokon *et al*., 2008) and one of such herbs is *Phyllanthus amarus* whose antiplasmodial activity has been reported (Onyesom *et al*., 2015). *Phyllanthus amarus* is commonly called "carry me seed" or "stone-breaker", it is known in Nigeria as "Oyomokeisoamankedem" in Etif, "Iyin Olobe" in Yoruba and "Ebebenizo" in Benin (Etta, 2008). The therapeutic properties of *P. amarus* have been linked to several phytochemicals including flavonoids (Obidike *et al*., 2013).

Phyllanthus amarus and the phytochemical flavonoid have been shown to be potent in the treatment and management of arrays of diseases and disorders, including malaria. However, knowledge of the inhibitory potency of *P. amarus* flavonoid on malarial parasite growth in key areas of the body *viz* blood, brain, liver and pancreas is not well documented. This study, therefore, investigates the changes in blood and tissue (brain, liver and pancreas) parasite load induced by the treatment of malaria with the ethanolic extract and flavonoid fraction of the stem of *P. amarus* in experimental mice.

Materials and Methods

Plant collection: Fresh plants of *Phyllanthus amarus* were collected from natural habitat in Abraka, Ethiope East Local Government Area of Delta State, Nigeria. The plant was identified at the Forestry Research Institute of Nigeria, Ibadan, Oyo State where a voucher specimen (No:FHI:109728) has been deposited.

Preparation of the ethanolic extract: The stems of the plant were collected, washed, air-dried and ground to produce a fine powder. Then, 100 g of the power was sequentially extracted with 1L of 95% ethanol using a soxhlet apparatus (Corning, USA). The extract was evaporated to dryness using rotary evaporator (Buchi R2 10, Henan, China) under reduced pressure and total yield obtained was 3.6% (3.6 g) which was dissolved in 50 mL of distilled water and divided into two portions of 25 mL each. One part was refrigerated at 4ºC and later administered as the ethanol extract, while, the other part was used to prepare the flavonoid fraction.

Extraction of flavonoid fraction: The 25 mL portion of the ethanol extract was introduced on a column (400 x 2.5cm i.d) packed with pre-treated AB-8 resin and after complete absorption of the solution, the column was washed with 50mL of distilled water to remove carbohydrates, and then, eluated with 100mL of 65% ethanol (Chen, 1998). The eluate, abundant in flavonoids, was collected and concentrated at 40ºC with laborata rotator evaporator (Heiddph, Schwabach, Germany) until the formation of sediment which was collected and vaccum-dried at 40ºC. The collected flavonoid (1.02 g) was dissolved in 20 mL of distilled water and stored in the refrigerator at 4ºC for use.

Determination of total flavonoids: The amount of total flavonoids in the preparation was determined by aluminium colorimetric method described by Zhishen *et al*. (1999). One hundred microlitre (100µL) of the already prepared flavonoid fraction was dispensed into a solution containing 0.7ml of 5% $NaNO_2$ and 10ml of 30% ethanol. This mixture was stirred for 5 min and 0.7ml of 10% aluminum trichloride was added and stirred for another 6 min. Then, 5ml of 40 mg/mL NaOH solution was added to the mixture and diluted to 25ml with 30% (v/v) ethanol. The mixture was allowed to stand for 10 min and the absorbance measured at 500 nm using a UV/Visible-spectrophotometer (UV/VIS SHIMADZU 1700, Henan, China). Quercetin was used as the standard and the total flavonoid content was expressed as quercetin equivalent in mg/mL.

Experimental Animals: Forty (40) adult male BALB/c mice, weighing between 20-30g were purchased from and also, kept in the Laboratory Animal Centre (LAC), of the Faculty of Basic Medical Sciences (FBMS), Delta State University (DELSU), Abraka, Nigeria. These animals were grouped into eight (8) with five mice in each group (n=5 mice/group) and used for the study. Three (3) *Plasmodium berghei*-infested (donor) mice were obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria, and were used to prepare inoculum used for the study.

Animal care and handling: The mice were fed on growers' mash obtained from Top Feeds Flour Mill, Sapele, Delta State, Nigeria, and were given clean drinking water *ad libitum*. The animals were kept in plastic cages, under controlled condition of 12h light/12h dark cycle. The animals were maintained in accordance with the guidelines provided by the Research and Bioethics Committee of the Faculty of Basic Medical Sciences, Delta State University, Abraka.

Animal grouping, inoculation and extract administration: The animals were caged into eight (8) groups (n=5/group). Group 1 (Negative control) had mice that were infested with *Plasmodium berghei* (chloroquine-sensitive NK65 strain) without treatment. Groups 2, 3 and 4 were infested, and treated with 150, 300 and 450 mg/kg/d flavonoid, respectively, while, Groups 5, 6 and 7 were also infested, but were treated with 150, 300, 450 mg/kg/d ethanolic extract, respectively. Then, Group 8 (Positive control) animals were infested and treated with 5 mg/kg/d chloroquine.

The mice were infested by obtaining parasitized blood (3 to 4 drops) from the cut tail tip of an infested (donor) mouse. Then, 0.1ml of infested blood was diluted in 0.9ml of phosphate buffered saline, PBS, pH 7.2 and the mice were injected with 0.1ml of the parasitized suspension containing about 12,000 parasites through the pineal region. Parasitaemia was confirmed after about 72 h using Giemsa stained thin blood films collected from the cut tail tip of the infested mouse and viewed under the microscope (TH-9845, Serico, China) at x100 magnification. The ethanolic extract of the stem of *P. amarus* and the flavonoid fraction, which were already prepared were administered as single daily dose using intragastric cannula for a period of seven (7) days, having confirmed parasitaemia. The volume of the ethanolic extract or flavonoid fraction administered was determined by the formula:

V (ml) = D (g/kg x B (kg)/C (g/ml) as stated by Erhirhie *et al*. (2014).

where D= Dose studied (g/kg b.wt),
B= Body weight (kg) of animal,
C= Concentration of the extract (g/ml) prepared, and
V= Volume (ml) of ethanolic extract or flavonoid fraction administered.

Collection of specimen: At the end of the 7-day treatment period, blood, brain, liver and pancreas specimens were collected from the mice after euthanizing on the 8th day in a chlorofoam (anaesthasia) chamber following an overnight fast.

Malaria parasite count: The malaria parasite count was determined by making a thin blood, liver, brain, or pancreas film on a clean grease free glass slide and left to dry. Thereafter, fixation was done by using methanol for 30 seconds and the slide was stained with Giemsa as described by Ochei and Kolhatkar (2000). The blood and tissue (liver, brain, pancreas) films were flooded with freshly diluted Giemsa stain for 30 min, then, washed off and the slides were air-dried on a draining rack. Thereafter, the dried smear was examined under the microscope (TH-9845, Serico, China) with oil emersion lens at x100 magnification.

The percentage suppression rates of plasmodial growth and multiplication was calculated using the formula:

Parasite suppression (%) = A- B/A x 100 (Ajala *et al*., 2011)

where A = Parasitaemia in untreated group
B = Parasitaemia in treated group

Statistical analysis: Experimental data were expressed as Mean±SD (Standard Deviation) for n= 3mice/group. Results were subjected to statistical analysis using one way analysis of variance (ANOVA) to determine the differences between groups. Differences between means at 5% level ($p<0.05$) were considered significant. Analysis was performed using IBM SPSS software version 20.

Results and Discussion

The results obtained from the investigation of the changes in parasite load induced by ethanolic extract and flavonoid fraction of the stem of *Phyllanthus amarus* in the blood and tissues of *Plasmodium berghei* infested mice are presented in Table 1. While, Table 2 shows the percentage suppression of parasite growth induced by the ethanolic extract and flavonoid fraction of the stem of *P. amarus* in the blood and tissue of the same infested mice.

Table 1: Parasite counts of the blood and tissues of *Plasmodium berghei* infested mice treated with various doses of ethanolic extract and flavonoid fraction of the stem of *Phyllanthus amarus*.

Group	Dose (mg/kg/d)	Blood	Brain	Liver	Pancreas
Parasite Count (%)					
1	0 (Control)	21.93±2.64[a]	21.41±0.25[a]	22.61±2.16[a]	4.66±0.19[a]
2	150 (Flavonoid)	7.58±0.39[b]	5.56±0.41[b]	6.07±0.80[b]	2.04±0.27[b]
3	300 (Flavonoid)	7.93±0.45[b]	7.06±0.82[b]	6.34±0.69[b]	1.67±0.04[b]
4	450 (Flavonoid)	7.87±0.26[b]	5.98±0.57[b]	7.81±0.39[b]	1.61±0.07[b]
5	150 (Extract)	2.44±0.47[b]	2.33±0.23[b]	2.49±0.10[b]	1.17±0.05[b]
6	300 (Extract)	2.63±0.02[b]	2.23±0.20[b]	2.53±0.08[b]	1.07±0.03[b]
7	450 (Extract)	2.21±0.22[b]	1.97±0.11[b]	2.34±0.20[b]	0.99±0.05[b]
8	5 (CQ)	2.65±0.29[b]	1.79±0.22[b]	2.26±0.23[b]	1.63±0.10[b]

Values that bear another superscript in a column differ significantly (p<0.05).
Values are expressed as Mean ± Standard Deviation (SD) for n = 3 mice per group.
CQ = Chloroquine

Table 2: Parasite suppression rates in blood, brain, liver and pancreas of *Plasmodium berghei* malarial parasite infested mice treated with varying doses of *P. amarus* ethanolic stem extract and flavonoid fraction

Tissue	Treatments						
	Flavonoid doses (mg/kg/d)			Stem extract doses (mg/kg/d)			CQ dose (mg/kg/d)
	150	300	450	150	300	450	5
	Changes in parasite suppression rates (%)						
Blood	64.78±5.59[a]	64.78±5.59[a]	63.65±3.96[a]	88.96±0.81[b]	87.94±1.37[b]	89.78±1.60[b]	87.59±2.61[b]
Brain	74.04±1.90[b]	67.03±3.78[a]	72.09±2.67[b]	89.13±1.18[b]	89.60±1.06[b]	90.77±0.61[b]	91.66±1.04[b]
Liver	72.61±5.89[a]	71.58±4.91[a]	65.13±3.90[b]	88.88±1.44[b]	88.71±1.38[b]	89.50±1.78[b]	90.03±0.49[b]
Pancreas	56.01±6.44[a]	64.27±1.13[b]	65.41±2.50[b]	74.85±0.95[b]	77.07±0.32[b]	78.63±1.70[b]	64.95±1.67[b]

Values that bear another superscript in a row differ significantly (p<0.05).
Values are expressed as Mean ± Standard Deviation (SD) for n =3 surviving mice per group.
CQ = Chloroquine

Malaria parasite has been associated with oxidative stress in red blood cells in blood, liver and brain tissues (Onyesom *et al*., 2015), and has also been known to affect pancreatic secretion of especially serum α-amylase and insulin (Intisar *et al*., 2014).

Mice model treatment of malaria infection with crude ethanolic extract of the stem of *Phyllanthus amarus* at 150, 300, and 450 mg/kg/d significantly (p<0.05) reduced parasite load in the blood, brain, liver and pancreas (Table 1), and also, suppressed parasite growth and multiplication in the blood and these tissues in a manner that compared very well with the standard chloroquine treatment (Table 2). However, suppressions by similar doses (150, 300 and 450 mg/kg/d) of the flavonoid fraction were moderate.

Results from this study show similarity with previous studies. At 100 mg/kg/d, significant (p<0.05) difference was observed between ethanolic extract of *Phyllanthus amarus* (1.20) and untreated group (1.66) in day 1 while day 11 was 0.40:4.00 (Nwaoguikpe *et al*., 2013), and the ethanolic *Phyllanthus amarus* extract showed no significant (p<0.05) difference from the standard Artemeter (100 mg/kg/d) treatment from Day 1 to day 11. In the same study, flavonoid was moderately present in the qualitative phytochemical screening. Ethanolic extract of *Phyllanthus amarus* (200, 400, 800 and 1600 mg/kg/d) demonstrated prophylactic and chemotherapeutic activity; the treatment also compare well with standard chloroquine (5 mg/kg/d) (Ajala *et al*., 2011).

Flavonoid fractions (200 and 400 mg/kg/d) of extract of *Adansonia digitata* showed chemosupression (Adeoye *et al*., 2016). Great deals of polyphenolic flavonoids that have been detected and isolated in dietary or medicinal plants have been identified to possess good *in vitro* and *in vivo* antimalarial activities (Lehane, 2008). Flavonoids are known to chelate nucleic acid base pairing of malaria parasites (Agrawal, 1989), and as phenolic compounds, they act as primary antioxidants or free radical scavengers (Ettebong *et al*., 2012).

It is evident from present study, that flavonoid-rich extracts of *P. amarus* stem has moderate suppressive effect in vital tissues (blood, brain, liver and pancreas) of malaria infested mice when compared with chloroquine standard treatment. The phytochemicals present in *P. amarus* are alkaloids, flavonoids, hydrolysable tannins, polyphenols and lignans and these phytocompounds have been linked to the chemoprotective and medicinal properties of the plant (Dhongade and Chandewar, 2014), this may explain the moderate chemosuppresion of flavonoids in present study as they are just a fraction of the whole *P. amarus'* antimalarial phytochemicals.
The crude ethanolic extract of *P. amarus* demonstrated profound antiplasmodial activity comparable to chloroquine, this might have been made possible due to the concerted activities of several phytochemicals in *P. amarus*. This similarity between the plant extract and chloroquine can further be explained by the presence of alkaloid in *P. amarus*. Chloroquine, 4-aminoquinoline is a synthetic derivative from the alkaloidal drug quinine (Uzor, 2020).
The findings of this study show the basis for the traditional use of *P. amarus* as antimalarial agent. They further reveal that flavonoid may be in synergy with other active phytochemical constituent(s) responsible for the significant antiplasmodial activity exhibited by the ethanolic extract of the stem of *P. amarus* as observed. Therefore, the active phytochemical constituent(s) should be identified in subsequent investigation, which could provide the pivot for new antimalarial drug discovery.

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