Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(4): 118-122

Received: 25-05-2017
Accepted: 26-06-2017

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In vivo antiplasmodial activities of ethanolic leaf extract and fractions of *Hilleria latifolia*

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Abstract
This study aimed at evaluating the *in vivo* antiplasmodial activities of the leaf extract and fractions (n-hexane, dichloromethane, ethyl acetate, butanol and aqueous) in *Plasmodium beighei beighei* infected mice**.** Mice were orally administered with the leaf extract (250, 500, and 750 mg/kg) of *Hilleria latifolia* and its fractions (500mmg/kg) and screened in the suppressive, repository and curative tests. Chloroquine and Artesunate (5 mg/kg) each and Pyrimethamine (1.2 mg/kg) were used as standard drugs. The extract showed significant (P< 0.001) dose-dependent, antiplasmodial activity in the suppressive, repository and curative tests and increased the survival times of the infected mice of the infected mice. All the fractions exhibited significant antiplasmodial activity with the highest being aqueous fraction. *Hilleria latifolia* extract and fractions possess antiplasnodial activity which confirms the ethnomedicinal use of this plant as a malarial remedy and opens a new highway to further investigate its potentials in the on-going fight against malaria.

Keywords: Antiplasmodial, Extract, *Hilleria latifolia*, *in vivo*

Introduction
Malaria is a mosquito-borne disease caused by the parasite, intraerythrocytic protozoa of the genus *Plasmodium* (e.g *P. falciparum, P. vivax, P. ovale,* and *P. malariae* among other species) [1]. It is a primordial disease that has been affecting human race since their origin. Albeit the parasite shows parallel divergence with hominids [2,3]. It has evolved so finely and shaped its genome to a great extent to invade, dodge and damage hosts defence system. Small generation time, pressure to survive and grow under adverse environmental conditions inside host, ability to disguise and escape host immune system; help the parasite to succeed the evolutionary arms race [3,4]. Malaria has continued to be an endemic disease of the tropics and subtropics, although endemic malaria has been documented in areas North and South of the equator, as far as latitudes 64^0N and 32^0. Hence, malaria associated morbidity and mortality is a major public health concern especially for underdeveloped and developing countries of the globe. Though many malaria control and eradication strategies have been followed since ages, but none of them are successful in an overall control program. In the absence of a competent vaccine for malaria prevention and at the same time emerging resistance against currently available antimalarials, the ongoing malaria control programs have been severely hampered. Besides that, cross resistance among drugs due to their alike chemical combination is also well evidenced [5]. As a result of which, the current malaria control program has been adversely affected by the development and spread of parasite resistant strains to the working antimalarial, ACT (Artemisinin-based Combination Therapy) [6].
Hilleria latifolia also known as *efehentok* in Ibibio, *aka ato* in Igbo language is a perennial plant with more or less woody stems that can persist. It can grow up to two meters tall. It bears simple, alternate leaves up to 15 cm long, 6cm which have alternate margins [7]. It is native to South-America, naturalized in tropical Africa and Sri-lanka [8]. Its habitat is mainly rainforest and groundwater forest [9].
Its ethno-medicinal uses include treatment of feverish conditions including malaria amongst several tribes in Nigeria. In Cote d'Ivoire, a decoction of the leaf is used to treat infective and inflammatory conditions such as urethral discharge and ear discharge. The crushed leaves are used in Congo as a lotion for different kinds of skin disorders including scabies and small pox [10]. Also in Ghana, a poultice of fresh leaves or roots is applied to boils. A leaf decoction, in small doses is given for the treatment of leprosy [10].

Previous works revealed the presence of alkaloids, triterpenoids, tannins and glycosides as secondary metabolites in all parts of the plant from phytochemical studies. Anti-inflammatory, anti-arthritic, anti-oxidant and Antimicrobial activity including *in vitro* antiplasmodial activity was also reported [10, 11, 12]. The present work seeks to evaluate via *in vivo* studies of the ethanolic leaf extract the pharmacological basis, if any, behind its ethnomedicinal use in the treatment of malaria.

Materials and Methods

Collection and Identification of Plant Materials.

The plant material (*Hilleria latifolia)* was collected from a private garden in Uyo, Akwa Ibom State, Nigeria. The plant was identified and authenticated by Professor (Mrs.) Margaret Bassey (a Plant Taxonomist) in the Department of Botany and Ecological Studies, Faculty of Science, University of Uyo, Akwa Ibom State, Nigeria. A voucher specimen (UUH3511) was deposited in the herbarium of the faculty. The plant was air-dried, oven- dried at reduced temperature and then pulverized into fine powder. The powder was extracted by cold maceration with 70% (v/v) ethanol at room temperature for 72 hours, and then filtered. The filtrate was then concentrated under reduced pressure at 40^0C using a rotary evaporator. Approximately, 30g of the extract was then dissolved in distilled water, then partitioned using various solvents in the following order: n-Hexane, Dichloromethane, Ethyl acetate, and Butanol respectively, the remainder constituted the Aqueous fraction. The crude extract and its fractions were stored in refrigerator at -4^0C until required for use.

Preliminary Phytochemical screening

The extract was screened for secondary metabolites such as saponins, alkaloids, tannins, flavonoids, anthraquinones and cardiac glycosides [14, 15].

Animal Stock

Adult albino mice (20 – 28g) were obtained from the Animal house of the University of Uyo, Akwa-Ibom State and fed with growers pellet feeds with water given *ad libitum.* Approval for the use of animals in the study was obtained from the Animals Ethics Committee, Faculty of Pharmacy, University of Uyo.

Micro-Organisms

A Chloroquine sensitive strain of *Plasmodium berghei berghei* (ANKA) was obtained from National Institute of Medical Research (NIMER) in Lagos and maintained by subpassage in mice.

Inoculum Preparation

The parasitized blood donor with high parasitaemia was obtained by first anaesthetizing the mouse with chloroform, and then through cardiac puncture, blood was obtained using sterile syringe into sterile heparinized bottle. The percentage parasitaemia was determined by counting the number of parasitized red blood cells against the total number of red blood cells. The desired volume of blood obtained from the donor mouse was suitably diluted with sterile normal saline so that the final inoculum (0.2 ml) for each mouse contained the required number of parasitized red blood cells. Therefore 0.2 ml of the final inoculum did contain 1.0×10^7 parasitized red blood cells which is the standard inoculum for the infection of a single mouse. 1 ml of the standard inoculum is expected to contain 5×10^{-7} parasitized red blood cells [15].

Drugs Administration

Drugs (Chloroquine powder, Pyrimethamine, and Artesunate), the extract and the fractions used in this study were administered through the oral route using stainless metallic feeding canulla.

Acute Toxicological Studies

Acute toxicological study was carried out to determine the median lethal dose (LD_{50}) using Lorke's method [16].

Antiplasmodial Activities of the Extract

Determination of Suppressive Activity of Extract (4 – Day Test)

The method as described by Knight and Peters with slight modification [17,18] was adopted. The mice were each inoculated on the first day (day 0), intraperitoneally with 0.2ml of infected blood containing *P. beighei berghei* parasitized erythrocytes. The animals were then randomly divided into six groups of six mice each. After ten minutes, the mice in the first group were orally administered 10mg/kg of distilled water and served as control. Group 2 – 4 received between 250 – 750 mg/kg of the extract orally. Group 5 and 6 received 5mg / kg/day of both Artesunate and Chloroquine as positive control respectively.

The administration of extract and drug was continued daily for 4 days (D0 – D3) between 8.00 – 9.00 a.m. On the fifth day (D4), thin blood film for malaria parasite was collected from the tail of each mouse directly to the labeled glass slides. The thin film slides were fixed with absolute methanol and then stained using Geimsa solution to reveal parasitized erythrocytes. The percentage parasitaemia was calculated by counting the number of parasitized red blood cells out of 500 erythrocytes in random fields of the microscope.

$$\% \text{ Parasitaemia} = \frac{\text{N}^{\text{o}} \text{ of parasitized RBC}}{\text{Total N}^{\text{o}} \text{ of RBCs counted}} \text{ x } 100$$

Average percentage chemosuppression was calculated by

$$100 \left(\frac{A-B}{A}\right)$$

Where A = Average % parasitaemia in negative control.
B = Average % parasitaemia in test group.

Determinaton of Antiplasmodial Activity of Fractions Using 4- Test

Using the same method as described above [17,18], various fractions of the extract (n- Hexane, Dichloromethane, ethyl acetate, butanol and aqueous) were administered orally at the dose of 400mg/kg/day to different groups of six mice/group 30 minutes after intraperitoneal injection of 0.2ml of infected blood containing about 1.0 x 107 *P. berghei berghei*. The negative control group received 10ml/kg of distilled water. The administration of fraction/distilled water was continued for 4 days (D0- D3). Thin films were prepared from the tail blood of each mouse on the fifth day and the level of parasitaemia was determined by counting the number of parasitized red blood cells out of 500 RBCs in random field of the microscope. The average parasitaemia was calculated as stated above.

Determination of Repository/Prophylactic Activities of Extract.

The method described by Ryley and Peters with slight

modification [18, 19, 20] was adopted. Mice were divided randomly into 5 groups of six animals per group. Group 1 animals received 10ml/kg of distilled water. Group 2 – 4 received 250 – 750 mg/kg of the extract orally. Group 5 animals (positive control) were administered 1.2mg/kg/day of pyrimethamine. All the groups were treated for 3 consecutive days (D0 – D2) and on day 4 (D3), the mice were intraperitoneally injected with 0.2mls of infected blood. The parasite density was assessed from thin films obtained from tail blood of each mouse within 72 hours of inoculation. The percentage parasitaemia and the average chemosuppression were calculated.

Determination of Effect of Extract on Established Infection (Curative or Rane test)

The method described by Lui *et al.* [18, 20] with slight modifications was adopted. Thirty mice were inoculated intraperitoneally with standard inoculums of 1 x 10^7 *Plasmodium berghei berghei* parasitized red blood cells on the first day (D0). Seventy two hours later, the mice were randomized into 5 groups of 6 animals per group. Group 1 received 10 ml/kg of distilled water. Group 2 – 4 animals were administered 250 – 750mg/kg/day of crude extract orally. Group 5 animals were administered with 5 mg/kg/day of chloroquine and artesunate. All the drugs and extract were repeated daily for 4 more days. Tail blood samples from each mouse were collected daily for 4 days, thin films was prepared and stained with Geimsa. The percentage parasitaemia and the average chemosuppression were calculated.

The Mean Survival Time (MST) of each group will be determined over a period of 30 days (D0 – D29).

$$\text{MST} = \frac{\text{No. of days survived}}{\text{Total No. of days (30)}} \times 100$$

Statistical Analysis

Results were expressed as multiple comparison of Mean ± SEM. Significance was determined using One-way Analysis of Variance (ANOVA) followed by Turkey-Kramer multiple comparisons post-test. A probability level of less than 5% was considered significant.

Results and Discussion

The plant *Hilleria latifolia* is used in the treatment of feverish conditions (of which malaria is one the most common causes) amongst the Ibibios and various other tribes in Nigeria and West Africa. This study was undertaken using *in-vivo* model in which the ethanolic leaf extract and its fractions were tested against *P. berghei berghei* infected mice. The *in-vivo* model was employed because it takes into account pro-drug effect and possible involvement of immune system in eradication of infection [21, 22].

The median lethal dose (LD_{50}) was determined to be 2,549.5mg/kg. The phytochemical screening of the leaf extract showed the following constituents were present: saponins, alkaloids, tannins, flavonoids, anthraquinones and cardiac glycosides, terpenes was absent. In the suppressive test (Table 1), there was a dose-dependent decrease in the levels of parasitaemia following the administration of the extract compared to the control. This decrease was statistically significant ($p < 0.001$). However the suppressive effect was less when compared with the standard drugs chloroquine and artesunate. The fractions also exhibited good chemosuppressive antiplasmodial activity with the aqueous fraction producing the highest effect. *In vitro* studies have shown antiplasmodial activity [11]. The fraction having the highest chemo suppressive effect was the aqueous fraction (64.43%) as shown in Table 2. The degree of suppression of *H. latifolia* was in the order: Aqueous > butanol > n-hexane > dichloromethane > ethyl acetate. This suggests that the bioactive secondary metabolites in the plant are mostly polar constituents. The 4-day suppressive test is a standard test commonly used for antimalarial screening [22, 23]. It is the most widely used preliminary test, in which the efficacy of a compound is assessed by comparison of blood parasitemia and mean survival time in treated and untreated mice [24] and the determination of percent suppression of parasitemia is the most reliable parameter [22, 23, 25].

Table 1: Antiplasmodial suppressive activity of extract

Drug/ Extract	Dose mg/kg	Parasitaemia (x10^7)	Chemosuppression/ %
Control	10	54.33 ± 2.38	
Extract	250	40.67± 0.76[a]	25.14
	500	32.33± 2.08[a]	40.49
	750	32.00± 2.92[a]	41.10
Artesunate	5	0.67± 0.42[a]	98.77
Chloroquine	5	0.33± 0.21[a]	99.39

Values are expressed as Mean ± S.E.M. Significance relative to control:
[a]$p < 0.001$; n= 6

Table 2: Antiplasmodial activities of fractions of extract using 4 – day test

Drug/ Extract	Dose mg/kg	Parasitaemia mg/kg	% Chemosuppression
Control	10	54.33 ± 2.38	
n-Hexane	500	24.33 ± 1.17[a]	51.00
Dichloromethane	500	28.66 ± 1.72[a]	42.28
Ethyl acetate	500	37.66 ± 2.20[a]	24.16
n-Butanol	500	22.33 ± 1.17[a]	55.03
Aqueous	500	17.66 ± 1.11[a]	64.43
Artesunate	5	0.67± 0.42[a]	98.77

Values are expressed as Mean ± S.E.M. Significance relative to control. [a]$p < 0.001$; n = 6.

An antimalarial compound is considered to be an active compound when its parasitaemic suppressive percentage is ≥ 30% [26]. This idea strongly supports the present study in which the extract and its fractions significantly suppressed

parasitemia level beyond this cut off point. Therefore, the fractions are active. Further, this signifies that agents with suppressive activity against *P. berghei berghei* were known for antimalarial activity [27]. In the repository test (Table 3), the extract showed dose-dependent decrease in parasitaemia and this decrease was statistically significant ($p < 0.001$) when compared to control.

Table 3: Antiplasmodial repository activity of extract.

Drug/ Extract	Dose mg/kg	Parasitaemia ($x10^7$)	Chemosuppression/ %
Control	10	41.33± 1.80[a]	
Extract	250	18.66± 0.55[a]	54.85
	500	13.67± 0.92[a]	66.92
	750	11.00± 0.73[a]	73.33
Pyrimethamine	1.2	3.66± 0.56[a]	91.14

Values are expressed as Mean ± S.E.M. Significance relative to control; [a]$p < 0.001$; n = 6.

There was a progressive dose and time dependent reduction in parasitaemia when the extract was tested on established infection (Table 4). The reductions were statistically significant ($p < 0.001$). The mean survival time of extract treated groups of mice were dose-dependently and significantly longer. The extract increased the mean survival time from 14 to 18 days when compared to the control. However, when compared to the standard drugs artesunate and chloroquine, the mean survival time was shorter (Table 5).

Table 4: Antiplasmodial activity of extract during established infection (Curative test)

Drug/ Extract	Dose mg/kg	Percentage Mean Reduction in Parasitaemia Per / Day			
		3	4	5	6
Control	10	45.00 ± 2.19	43.00 ± 4.78	38.00 ± 0.96	32.00 ± 1.46
Extract	250	29.00 ± 1.81	21.00 ± 0.73	16.33 ± 1.47	12.00 ± 1.31
	500	22.00 ± 1.31	17.33 ± 0.55	14.00 ± 0.73	9.00 ± 0.96
	750	14.00 ± 1.59	13.33 ± 1.47	11.33 ± 0.92	7.66 ± 0.76
Artesunate	5	1.33 ± 0.55	1.00 ± 0.36	0.16 ± 0.16	0.33 ± 0.21
Chloroquine	5	0.33 ± 0.21	0.50 ± 0.22	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as Mean ± SEM. Significance relative to control; [a]$p < 0.001$. n = 6.

Table 5: Mean Survival Test (MST) of mice receiving various doses of extract.

Drug/ Extract	Dose mg/kg	MST (Days)
Control	10	13.80 ± 1.53
Extract	250	17.66 ± 1.28[a]
	500	18.30 ± 1.30[a]
	750	20.00 ± 2.56[a]
Artesunate	5	26.66 ± 1.28[a]
Chloroquine	5	28.33 ± 0.66[a]

(Mean ± SEM), n = 6, Significance relative to control [a]$p < 0.001$.

The evaluation of the leaf extract of *Hilleria latifolia* and its fractions showed that the plant has a great potential as an antimalarial agent as observed in its suppressive, repository and curative activity against mice infected with *P. berghei berghei*. In the 4-day test, there was a dose-dependent decrease in the levels of parasitaemia. This decrease, though statistically significant, was less when compared with the standard drugs.

In the repository test, the extract also exhibited statistically significant, dose-dependent chemosuppression in all the treated groups with the highest effect observed in the group treated with high dose 750 mg/kg. The extract exerted a higher chemosuppressive effect in the prophylactic model than in the 4-day test. There was minor chemo suppressive difference observed between the high dose (750 mg/kg) and the middle dose (500 mg/kg) in both the 4-day (1%) and the prophylactic (7%) tests as compared to the difference between the low and middle dose. This suggests that 500 mg/kg may be the optimum therapeutic dose in mice.

In established infection, the extract exerted significant progressive and dose-dependent parasitaemia. The highest reductions occurred on day 6 when compared to control. However, the curative effect was greatest with greatest with Chloroquine, followed by Artesunate. In addition to the significant suppressive effect on parasitemia, the extract also improved the survival time of the mice relative to the control. This finding probably indicates that the extract suppresses *P. berghei berghei* and reduces the overall pathologic effect of the parasite in mice. The observed antiplasmodial activity may be associated with the presence of secondary metabolites such as alkaloids and flavonoids [12]. Alkaloids have been known have been known to exibit antiplasmodial potentials by blocking protein synthesis in *Plasmodium falciparum.* Flavonoids have been reported to chelate the nuclei acid base pairing of the parasite [28]. The oral median lethal dose value for the ethanol leaf extract of *H. latifolia* obtained in mice was found to be non-toxic. The Organization for Economic Cooperation and Development (OECD), Paris, France, recommended chemical labeling and classification of acute systemic toxicity based on oral median lethal dose values as: very toxic if ≤ 5 mg/kg; toxic if > 5 mg/kg but ≤ 50 mg/kg; harmful if > 50 mg/kg but ≤ 500 mg/kg and non-toxic or not harmful if > 500 mg/kg or ≤ 2000 mg/kg [29]. The results of this study justify as well as confirm its ethnomedicinal use in the treatment of malaria amongst the different ethnicities in Nigeria.

Conclusion

This study shows that *H. latifolia* contains secondary metabolites (alkaloids, flavonoids, steroids, cardiac glycosides, tannins etc.) which may be responsible for its observed suppressive, repository and curative antiplasmodial activities thereby lending credence for its ethnobotanical use in the treatment of various feverish conditions of which malaria is a common cause. Toxicological studies show that the leaf is relatively safe. *H. latifolia* may be of value in the development of new therapy for treatment of malaria.

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