An *in vivo* antiplasmodial activity of aqueous and ethanol whole plant extracts of *Phyllanthus fraternus* using *plasmodium berghei* infected balb/c mice.

Keywords

In vivo, antiplasmodial activity, *Phyllanthus fraternus*, phytochemicals, percentage chemosupression, percentage parasitemia, *Plasmodium beighei* and *Balb/c mice.*

Abstract

Background: Medicinal plants represent potential sources for the discovery of new antimalarial agents. The *in vivo* anti-plasmodial activity of aqueous and ethanol whole plant extracts of *P. fraternus* were evaluated using *Plasmodium beighei* infected *Balb/c* mice.

Methodology: The extracts were screened for their phytochemical constituents to show the presence of secondary metabolites. The LD_{50} of both extracts were investigated and found to be greater than 5000 mg/kg. The *in vivo* antiplasmodial activity (percentage parasitemia (%P) and the percentage chemo-suppression (%C)) of the extracts were evaluated using animal model.

Results: The aqueous and ethanol extracts established modest antiplasmodial activity in a dose dependent manner (Table 3). The standard drug (coartem 2 mg/kg) with percentage parasitaemia of 28.57±4.70 and 2.48±0.48 caused percentage chemosuppression of 44.38±7.63 and 81.27±2.07 in day four and six respectively. From (figure 1 and 2), the test groups (aqueous and ethanol extracts) for two different doses (100 mg/kg and 200 mg/kg) each administered with percentage parasitemia 39.67±1.35, 39.58±1.64, 37.32±2.37, 36.23±1.99 and 10.24±1.32, 9.33±0.66, 8.61±0.96, 7.27±1.26 caused percentage chemosuppressions of 22.78±2.20, 22.96±2.66, 27.35±3.84, 29.48±3.23 and 22.54± 9.93, 29.43±4.99, 34.87±6.66, 44.99 ±5.98 in day four and six respectively. The aqueous extract

demonstrated better inhibition of *plasmodium* in doses 100 mg/kg and 200 mg/kg with chemosuppressions (27.35 ± 3.84 and 29.48 ± 3.23) respectively compared with the ethanol extract of the same doses 100 mg/kg and 200 mg/kg with chemosuppressions (22.78 ± 2.20 and 22.96 ± 2.66) respectively. The activity of the standard drug, coartem at 2.0 mg/kg was significantly higher ($P< 0.05$) with chemosuppression (44.38±7.63) than those of the extracts. The extracts were also screened for phytochemicals for which some were found in the extracts which have previously been implicated as antiplasmodial agents. The LD_{50} of both extracts were investigated and found to be greater than 5000 mg/kg.

Conclusion: The aqueous and ethanol whole plant extracts of *Phyllanthus fraternus* possesses antiplasmodial activity and would be useful in the search for novel antimalarial agents.

Background

The plant *Phyllanthus fraternus* belongs to the Family *Euphorbiaceae* and is commonly called gulf leaf-flower, Chancapiedra, stone breaker, carry-me-seed, hurricane weed or quinine weed. The plant also has local names such as Mache da goyo (Hausa), Gbogbonowun lese (Yoruba), Ofobi okpabi (Krobo and Ga), Lume or Kpavideme (Ewe), Awommaaguwakyi (Twi) [1]. It is an annual dicotyledonous herb which is small, erect and grow in gutters, dumping places and along the road of 30 to 40 cm in height [2]. Traditional herbalist in Ghana uses the whole plant for numerous pharmacological activities such as blennorrhagia, colic, diabetes, dysentery, fever, flu, tumors, jaundice, vaginitis, and dyspepsia [3]. From literature *Phyllanthus fraternus* possesses anti-inflammatory [1], antioxidant and anticoagulant [2], antidiabetic [4-5], antiviral [6] and analgesic properties [7-8].Through bites of Anopheles mosquitoes a parasite called *Plasmodium* species are transmitted into human which generate a malaria disease [9]. Antiplasmodial activity of different species of the genus phyllanthus have been determined elsewhere [10], but as far as literature can tell no work

have been done on an *in-vivo* of the aqueous and ethanol whole plant extracts of *Phyllanthus fraternus* against malaria. As a matter of fact, the existing orthodox drugs have lots of side effects and the most efficacious among them are now becoming impotent to the parasite and there is a need to research on new antimalarial plants (*Phyllanthus fraternus*) [1].

P. fraternus using *Plasmodium berghei* infected balb/c mice were employed in this study because they have similar properties of genetics, anatomy and physiology with humans in terms of experimental research. The types of mammals normally used for animal model experiments are rodents and these include; rats, mice, gerbils, guinea pigs and hamsters. Especially mice are used due to their similarity of genomes that mimics humans and also their cost effective [11]. João *et al.,* indicate that infected male Balb/c mice (20–28 g, n = 10/group) with *Plasmodium berghei* Anka parasite erythrocytes (PRBC 106) were treated orally with chloroquine as control [12]. Methanol extract of *B. orellana* was assessed on hepatoprotective activity against carbon tetrachloride induced hepatotoxicity in albino rats and it was established that 500 mg/kg of body weight made a reduction of 52.08%, 57.37% and 52.90% of serum level of ALT, AST and cholesterol respectively [13]. Barret et al., conducted an *in vitro* study to evaluate the efficacy of quinacrine using animal model and it was observed to be ineffective based on the conditions employed [14]. The combination of flowers of *Z. scabra* and other herbal medicines are used for topical treatment of alopecia, wound and eczema [15]. The Scientific proofs to back these claims are almost destitute with an exception of few works conducted on antimicrobial activity for the dried powdered leaves of *Z. scabra* for which *Phyllanthus fraternus* is not an exception [16, 17]. Even though there is an advancement into modern medicines, countries under developed rely massively on medicinal plants for their survival during disease attack. To get rid of malaria infection in the countries under developed, the World Health Organization aimed to include traditional medicine for its preventive approached. Many medicinal plant have been employed as

antimalarial properties by traditional herbalists but their effectiveness have not been scientifically assessed [18]. The Herbalist in Ghana documented the plant *Phyllanthus fraternus* as antimalarial drug but as far as literature can ascertain, it had not been scientifically assessed. There was no much adverse effects assigned to medicinal plants since its existence and are also believed to be significant in terms of new source of chemical substances with a therapeutic effects. Therefore the aim of the study is to evaluate an *in vivo* antiplasmodial activity of aqueous and ethanol whole plant extracts of *P. fraternus* using *Plasmodium berghei* infected balb/c mice.

Materials and Methods

Drugs and chemicals

All drugs and chemicals used such as tetraoxosulphate (vi) acid (H_2SO_4), ammonium hydroxide (NH_3OH (aq)), magnesium ribbon, 2 mL of hydrochloric acid (HCl), chloroform, ammonia, ferric chloride, acetone, sodium picrate paper, fehling solution A and B, 70% ethanol, giemsa stain, methanol and sodium chloride, were obtained from British Drug House Ltd (Poole, England). Coartem was obtained from Troge Medical GMBH (Hamburg Germany) were all of analytical grade unless otherwise stated.

Plant Raw Materials and Herbal Standard

Phyllanthus fraternus whole plant material (leaves, stems and roots) were obtained from the Plant Production Department (PPD), of Centre for Scientific Research into Plant Medicine (CSRPM) Mampong-Akuapem, Ghana and authenticated by Dr. Yaw Ameyaw, a botanist of the production department.

Animals

Seven-week old female balb/c mice (30 g) were obtained from the animal unit of the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem, in the Eastern

Region of Ghana. The animals were fed on powdered feed obtained from Ghana Agro Food Company (GAFCO), Tema, Ghana. They were allowed free access to sterile distilled water.

Preparation of Herbal Extracts

The plant material was cut into small pieces and spread thinly on a flat clean tray to prevent spoilage by moisture condensation and allowed to dry at room temperature for three (3) days. The dried plant material (195 g) was boiled in 4 L of water for 30 minutes and cooled. The resultant extract was filtered through a cotton wool and put in an oven at 50 ^{O}C to concentrate it before it was pre- freeze and lyophilized into powder using a freeze dryer (Heto powder dry LL 300, Sapa). The dry powder was weighed to determine the yield and stored in a desiccator at room temperature. This was reconstituted in sterilized distilled water before use. 70% ethanol extract was obtained by simple maceration of 195 g of dried sample of whole plant of *Phyllanthus fraternus* in 2 L aqueous ethanol (1.4 L of ethanol plus 0.6 L of distilled water) for 72 h. It was filtered through cotton wool and subjected to rotary evaporator (ILA CCA-1111 Japanese branch) to evaporate the ethanol and then pre-freeze and freeze- dried.

Malaria parasites and inoculum preparation

Plasmodium berghei NK65 strain from the University of Copenhagen Denmark through the Department of Immunology, Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, Accra, Ghana, was used for the experiment. The stock of parasitized erythrocytes was obtained from infected balb/c mice, with a minimum peripheral parasitemia of 20%, by cardiac puncture in heparin-coated tube. The cell concentration of the stock was determined and diluted with physiological saline such that 0.2 mL of final inoculum contained 10^6 parasitized red blood cells (RBCs).

Acute toxicity test

The acute oral toxicity study was conducted to know the amount of dose to be given to the animals. This was done by the Organization for Co-operation and Development (OECD) guidelines 425 received from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) [11].

Treatment of Animals

Thirty six (36) mice were selected and put into six (6) groups of six per group. Each mouse was inoculated intraperitoneal with the parasite *Plasmodium berghei*. Group 1 (Gp1) animals received distilled water (negative control), group 2 (Gp 2) animals received 5 mg Coartem (positive drug control), group 3 (Gp 3) and group (Gp 4) animals received 100 mg/kg and 200 mg/kg of ethanol extract of whole plant of *Phyllanthus fraternus* respectively, group 5 (Gp 5) and group 6 (Gp6) animals received 100 mg/kg and 200 mg/kg aqueous extract of whole plant of *Phyllanthus fraternus* respectively. All the drugs were orally administered to the animals (0.2 mL) for 2-3 h after the mice have been inoculated with the parasite over a period of 6 days.

Monitoring of Parasitemia and Antimalarial Activity

On the fourth and sixth days after drug administration, thin blood smears were prepared using blood from the tail vein of each mouse. Each smear was air-dried, fixed in methanol, air-dried again, stained with giemsa for 10-15 minutes and examined under oil immersion with a microscope. Each slide was observed at three different fields and the Red Blood Cells (RBC_S) and total number of RBC_S for each field was recorded. The percentage parasitemia (% P) and the percentage chemo-suppression (% C) also known as the activity was estimated according to the following formulae $\% P = 100 \times \frac{PRBC - TRBC}{TRBC}$ $\% C = 100 \times \frac{PCON - PTEST}{PCON}$

Where; $PRBC$ is the number of parasitized Red Blood Cell (RBC).

$TRBC$ is the total number of RBC counted per field.

PCON is the control parasitemia and PTEST is the test parasitemia.

Statistical analysis

Data were presented as means ± SEM of n= 6 and analyzed by using One-way ANOVA which was followed by students t-test. The $p \leq 0.05$ was considered statistically significant in all analysis.

Results

Phytochemical screening was carried out for aqueous and ethanol whole plant extracts of *Phyllanthus fraternus* which identified the presence and absence of groups of secondary metabolites using the standard method [19-20].The phytochemical screening of the extracts showed the presence of alkaloids, saponin, phenolics, reducing sugars, triterpenes and phytosterols in both extracts while cynogenic glycoside and anthraquinones were absent in both extracts and flavonoids and polyuronides showed presence only in the aqueous extract (Table 1).

Table 1: Phytochemical constituents of *Phyllanthus fraternus* whole plant extracts.

PHYTOCHEMICAL	EXTRACTS	
	Aqueous	Ethanol
Alkaloids	+	+
Saponins	+	+
Phenolics	+	+
Reducing Sugar	+	+
Polyuronide	+	-
Terpenoids	+	+
Flavonoids	+	-

Phytosterols	+	+
Anhthracenoside	-	-
Cyanogenic Glycoside	-	-

(+) = Present and (-) = Absent

Acute toxicity test

The LD_{50} of the extracts were identified and was greater than 5000 mg/kg and may be classified as practically non-toxic and within the acceptable margin of safety (Hodge and Sterner scale) at the recommended dose. Thus $1/50^{th}$ and $1/25^{th}$ (i.e.100 mg/kg and 200 mg/kg) were selected for the study (Table 2).

Table 2: Acute toxicity test for *Phyllanthus fraternus* whole plant of aqueous and ethanol extracts

	***Phyllanthus fraternus* whole plant**	
	Aqueous extract	**Ethanol extract**
Species and strain	Sprague-Dawley rats	Sprague-Dawley rats
Number of animals	Twelve (12)	Twelve (12)
Sex	Females	Females
Number. of groups	3 (N=4)	3 (N=4)
Route of administration	Oral	Oral
Formulation	Freeze dried	Freeze dried
Dose administered (mg/kg)	1250, 2500, 5000	1250, 2500, 5000
Period of observation	48 hours	48 hours
Number. of deaths	Zero (0)	Zero (0)
Approximate lethal dose(LD_{50})	>5000 mg/kg	>5000 mg/kg

Signs of toxicity	Nil	Nil

Percentages of Parasitemia and Chemo-suppression of *Phyllantus fraternus* whole plant of aqueous and ethanol extracts in 4th and 6th days test

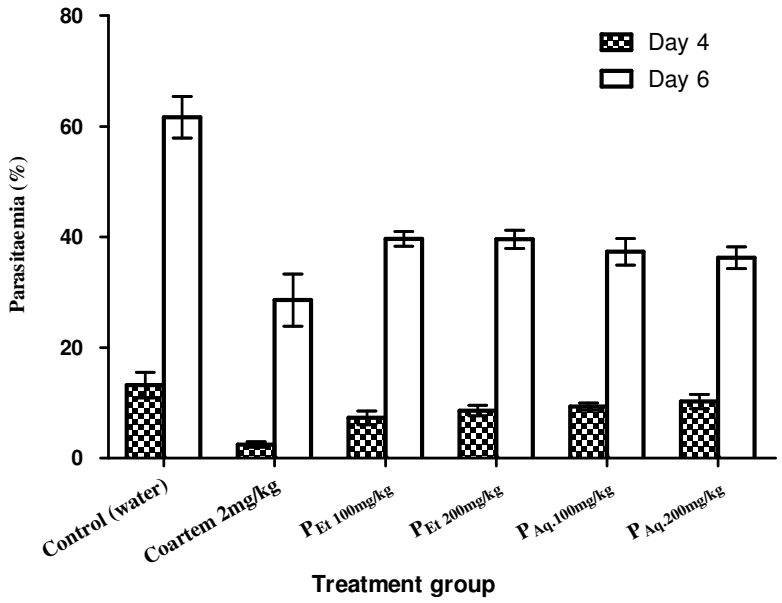
The route of administration of the controls (Coartem and distilled water) at doses of 2 mg/kg orally; aqueous and ethanol whole plant extracts of *Phyllanthus fraternus* were given orally at doses of 100 mg/kg and 200 mg/kg which significantly exerted *in vivo* antiplasmodial activity on the *Plasmodium berghei* induced in balb/c mice in a dose-dependent fashion (Table 3).

Table 3: Results of Percentage Parasitemia and Chemosuppression of 4 and 6 days test

Extracts	Day four		Day six	
Concentration (mg/kg)	Parasitemia (%)	Chemosuppression (%)	Parasitemia (%)	Chemosuppression (%)
Control	61.64±3.77	0.00	13.22±2.32	0.00
Coartem 2	28.57±4.70	44.38±7.63	2.48±0.48	81.27±2.07
PET 100	36.23±1.99	29.48±3.23	10.24±1.32	22.54±9.93
PET 200	37.32±2.37	27.35±3.84	9.33±0.66	29.43±4.99
PAQ 100	37.32±2.37	27.35±3.84	10.24±1.32	34.87±6.66
PAQ 200	39.67±1.35	22.78±2.20	7.27±1.26	44.99±5.98

Graphs of Percentages of Parasitemia and Chemo-suppression of *Phyllantus fraternus* whole plant of aqueous and ethanol extracts in 4th and 6thdays test

The results obtained from Percentage Parasitemia and Chemo-suppression of 4 and 6 days test (Table 3) were represented graphically where PAq = Aqueous extract of *Phyllanthus fraterus,* PEt = Ethanol extract of *Phyllanthus fraternus,* Results are means ± SEM of n= 6, * = Values significantly different from Distilled water controls ($p<0.050$) and # = Value significantly different from positive controls ($p<0.050$) (Figure 1 and 2).



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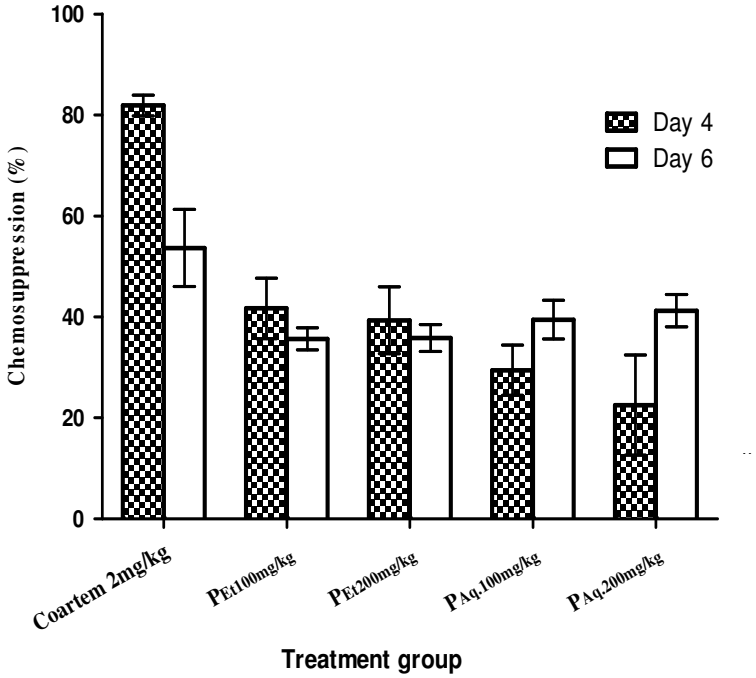
195 Figure1: Graph of the percentage parasitemia of *Plasmodium berghei* infected balb/c mice at
196 day four and six.



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198 Figure 2: Graph showing the percentage Chemo-suppression of *Plasmodium berghei* infected
199 *balb/c* mice at day four and six.
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Discussions

The phytochemical screening of the extracts showed the presence of alkaloids, saponin, phenolics, reducing sugars, triterpenes and phytosterols in both extracts while cynogenic glycoside and anthraquinones were absent in both extracts and flavonoids and polyuronides showed presence only in the aqueous extract. The result showed differences from reported works by [21-22]. The factors attributed to these differences were as a result of environment of the plant, mode of extraction and the climatic conditions [23-24]. Reports have shown that antiplasmodial activity of many agents were due to interference with the reproductive system of the protozoa [25]. Several reports have implicated alkaloids [26], terpenoids [27] and lignans [28-30] as antimalarial agents. The antiplasmodial activity demonstrated by both extracts may be attributed to the present of some of these phytochemicals. The extracts showed modest antiplasmodial activity in a dose dependent manner as manifested in the results from (Table 3). The standard drug (coartem 2 mg/kg) with percentage parasitemia of 28.57±4.70 and 2.48±0.48 caused percentage chemosuppression of 44.38±7.63 and 81.27±2.07 in day four and six respectively. From (figure 1 and 2), the test groups (aqueous and ethanol extracts) for two different doses (100 mg/kg and 200 mg/kg) each administered with percentage parasitemia 39.67±1.35, 39.58±1.64, 37.32±2.37, 36.23± 1.99 and 10.24±1.32, 9.33±0.66, 8.61±0.96, 7.27±1.26 caused percentage chemosuppressions of 22.78±2.20, 22.96±2.66, 27.35± 3.84, 29.48±3.23 and 22.54±9.93, 29.43±4.99, 34.87±6.66, 44.99± 5.98 in day four and six respectively. The plant *Phyllanthus fraternus* was observed to show intrinsic antiplasmodial activity by its percentage chemosuppressions (figure 2) and even curative ability as compared to that of the standard drug (coartem) but the relatively higher potency of the standard drug (coartem) was not surprising since it is a first line drug used in treatment of malaria, its active constituents are in refined state as compared to the crude extracts of the plants [31-33]. Generally, the low antiplasmodial activity could be

attributed to the crude nature of the extracts. The result (table 3) showed that the aqueous extract work best than the ethanol extract by increasing the concentrations of both extracts. The low percentage chemosuppression of the ethanol extract could be as a result of the poor solubility nature of the active components in the organic solvent and also the extract contain possible antagonistic compounds that hinders the activity of the active ones and increasing the concentration of the extract also increases the antagonistic components thereby reducing the activity of the extract. Further investigations are warranted to ascertain the exact mechanisms by which *Phyllanthus fraternus* aqueous extract exerts these effects. Nevertheless, these findings lend some information to the use of *Phyllanthus fraternus* aqueous and ethanol extracts in the management of antiplasmodial activity.

Conclusion

Phyllanthus fraternus aqueous and ethanol whole plant extracts from the results exhibited antiplasmodial activity, thus supporting its traditional uses in the management of malaria. A product formulated from the plant could be beneficial as adjunct therapy for management of *plasmodial* infections in Ghana.

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