Antiplasmodial activity of leaf extract and fractions of *Rutidea smithii* Hiern (Rubiaceae)

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ABSTRACT

Background: Resistance to malaria infection has been of great public health concern all over the world especially in developing countries thus there is need for development of new antimalarial drugs. Natural products including medicinal plants are veritable sources for drug discovery hence their investigation for possible antimalarial effects. *Rutidea smithii* is a woody climbing plant used traditionally in treatment of headache, cold and fever.

Objective: To investigate the *in vivo* antimalarial activity of ethanol leaf extract and fractions of *Rutidea smithii* in chloroquine sensitive *Plasmodium berghei* infected mice.

Methods: Swiss albino mice were intraperitoneally infected with chloroquine sensitive *P. berghei* (ANKA strain). The mice were treated orally using suppressive and curative models with graded doses of extract of *R. smithii* (100, 200 and 400 mg/kg), fractions (50, 100 and 200 mg/kg) and standard antimalarial drug; chloroquine (10 mg/kg). Preliminary phytochemical screening and acute toxicity study were also carried out.

Results: In the suppressive and curative tests, the extract demonstrated significant dose reduction in parasite level ($p < 0.05$) in infected mice and the survival time was also prolonged. The antimalarial activity of the fractions increased in the order hexane < butanol < aqueous < ethylacetate. The extract was devoid of toxicity up to the highest dose tested (2000 mg/kg).

Conclusion: *Rutidea smithii* has potent *in vivo* antiplasmodial activity against *P. berghe*i which resides mainly in ethylacetate fraction thus is a veritable source of new antimalarial agents.

Keywords: *Rutidea smithii*; Antimalarial; Curative; Suppressive; Ethylacetate fraction

Activité antiplasmodique de l'extrait de feuille et des fractions de *Rutidea smithii* Hiern (Rubiaceae)

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RESUME

Contexte : La résistance à l'infection par le paludisme a constitué un grave problème de santé publique dans le monde entier, en particulier dans les pays en développement. Il est donc nécessaire de mettre au point de nouveaux médicaments antipaludiques. Les produits naturels, y compris les plantes médicinales, sont de véritables sources de découverte de médicaments, d'où leur examen pour des éventuels effets antipaludiques. *Rutidea smithii* est une plante grimpante ligneuse utilisée traditionnellement pour le traitement des maux de tête, du rhume et de la fièvre.

Objectif : étudier l'activité antipaludique *in vivo* de l'extrait de feuille d'éthanol et des fractions de *Rutidea smithii* chez des souris infectées par *Plasmodium berghei* sensibles à la chloroquine.

Méthodes : Des souris albinos suisses ont été infectées par voie intrapéritonéale avec *P. berghei* (souche ANKA) sensible à la chloroquine. Les souris ont été traitées par voie orale en utilisant des modèles suppressifs et curatifs avec des doses graduées d'extrait de *R. smithii* (100, 200 and 400 mg/kg), de fractions (50, 100 and 200 mg/kg) et d'un antipaludique standard ; chloroquine (10 mg/kg). Un dépistage phyto-chimique préliminaire et une étude de toxicité aiguë ont également été réalisés.

Résultats : Dans les tests suppressifs et curatifs, l'extrait a démontré une réduction significative de la dose de parasite ($p < 0.05$) chez les souris infectées et la durée de survie était également prolongée. L'activité antipaludique des fractions augmentait dans l'ordre hexane < butanol < aqueux < acétate d'éthyle. L'extrait était dépourvu de toxicité jusqu'à la dose la plus élevée testée (2000 mg/kg).

Conclusion : *Rutidea smithii* possède une puissante activité antiplasmodique *in vivo* contre *P. berghe*i qui résident principalement dans la fraction d'acétate d'éthyle, ce qui en fait une véritable source de nouveaux agents antipaludiques.

Mots-clés : *Rutidea smithii*; Antipaludique; Curatif; Suppressive; Fraction d'acétate d'éthyle

INTRODUCTION:

Malaria is a major parasitic disease in the world especially in tropical and subtropical regions. According to World Health Organization (WHO), it is responsible for 2 to 3 million deaths every year, mostly children under five years and pregnant women.[1-2]

In Nigeria, malaria is the commonest cause of hospital attendance in all age groups. It is estimated that 30 percent of the population has at least one episode each year while children have an average of 3 to 4 attacks in a year.[3] Resistance of *Plasmodium strains* against the widely available antimalarial drugs like artemisinin and chloroquine makes the search for new agents a necessity.[4] Natural products have played a significant role in the discovery and development of drugs to treat human diseases. Many people in Africa still depend on traditional medicines for primary health care .[5] Medicinal plants are widely used in the continent in treatment of malaria and other ailments however there is little research on the plant extracts to establish their efficacy.

Rutidea smithii is a scandent shrub or woody climbing plant that grows up to 6 m long in the secondary jungle or rain forest margins in many West and East African countries including Nigeria. The thin twigs of the plant are hollowed and used in Kenya as drinking straws. The leaves have an unpleasant strong and sharp flavor. The powdered leaf is applied locally to wounds and used as nasal decongestant in cases of headache and cold in Ivory Coast.[6] Interaction with some traditional healers from South West Nigeria revealed that the leaf decoction of *R. smithii* was used in treatment of malaria. The literature search revealed very little information about the plant. The objective of this study was to investigate the antimalarial effect of ethanol extract and fractions of leaves of *R. smithii* in mice infected with chloroquine sensitive strain of *P. berghei*.

METHODS

Collection and preparation of plant material

The fresh leaves of *R. smithii* were collected in July 2015 from Aba Arokun village in Ikare, Ekiti State, Nigeria. The taxonomical identification of the plant was done by Mr. Oyebanji Oyetola of Herbarium unit, Department of Botany, University of Lagos, Nigeria, after comparing with herbarium specimen with voucher number LUH 5455. The leaves were cleaned and oven dried at 40°C then ground to coarse powder using grinder (Hamburg 76 West Germany).

Extraction and fractionation of extract

A total of (800 g) dried leaves were extracted by maceration with 5 L of absolute ethanol for 72 h with repeated agitation and filtered. The filtrate was dried using rotary evaporator at temperature of 40°C. The extract (30 g) was suspended in 400 mL distilled water and partitioned gradiently into n-hexane, ethylacetate and butanol (3 x 400 mL each) to obtain various fractions that were concentrated and dried. The dried extract and fractions were kept at 4°C until required for use.[7]

Experimental animals

Swiss albino mice of both sexes (20-22 g) procured from Laboratory Animal Center, College of Medicine, University of Lagos, Lagos, Nigeria were used for the *in vivo* antimalarial study. They were maintained at temperature of 25 ± 3°C and 12 h light /12 h dark cycle with food and water *ad libtum* in the animal house. The animals were allowed to acclimatize for 7 days prior to the experiments. The care and handling of the animals was according to international guidelines for use and maintenance of animals for research as approved by Health Research Ethics Committee College of Medicine of the University of Lagos, Nigeria with protocol ID number CM/HREC/12/16/086.

Acute toxicity studies

The acute toxicity test of the extract and fractions was determined according to OECD guideline No 420 (Organization of Economic Co-operation and Development). Mice (both sex) were used for this study. After the sighting study, a starting dose of 2000 mg/kg of the test samples were given to various groups containing 5 mice in each group. The treated animals were monitored for 14 days for mortality and general behavior.[8]

Parasite infection

Chloroquine sensitive strain of *Plasmodium berghei* (ANKA strain) used for the study was obtained from Nigerian Institute of Medical Research (NIMR), Lagos, Nigeria and was maintained by continuous re-infection in mice. Parasitized erythrocytes were obtained from donor infected mouse by cardiac puncture. The blood collected was diluted with phosphate buffer saline (PBS) based on parasitaemia level of the donor and red blood cell count of the normal mice such that 1 mL blood contained 5 x 10^7 parasites ensuring infected blood suspension of (0.2 mL) contained 1x10^7 parasitized red blood cells.

Suppressive test

The suppressive activities of the extract and its fractions were evaluated in early *P. berghei* infection in mice using the method of Knight and Peters.[9] Twenty-five mice were divided into five groups of five mice each. On the first day (D0), the mice were inoculated intraperitoneally with 0.2 mL of infected blood containing 10^7 *P. berghei*. Three hours later, group 1 received 0.2 mL of 1% DMSO (negative control), groups 2, 3 and 4 received 100, 200 and 400 mg/kg of leaf extract respectively while group 5 received 20 mg/kg chloroquine phosphate orally. The mice were treated separately for four consecutive days (D0-D3).

On the fifth day (D4), thin blood film was prepared from the tail of each mouse, fixed with methanol and stained with 10% Giemsa to show the parasitized erythrocytes. The parasite level was determined using microscope and the equation below:

$$\% \text{ Parasitemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC}} \times 100$$

$$\% \text{ Suppression} = \frac{A - B}{A} \times 100$$

Where A is the percentage parasitemia in the negative control group and B is the percentage parasitemia in the test group.

The mean survival time for each group was calculated by finding the average survival time (days) of the mice in each group over a period of 28 days (D0-D27). The experiment was repeated following the same procedures for various fractions of the extract at 50, 100 and 200 mg/kg.

Curative test

The curative effect of the extract and most potent fraction were determined using Ryley and Peter's method.[10] On the first day (D0), twenty-five mice were inoculated intraperitoneally with 10^7 infected erythrocytes and 72 h later the mice were divided into 5 groups of 5 mice per cage. Group 1 received 0.2 mL 1% DMSO, group 2, 3 and 4 received 100, 200 and 400 mg/kg of leaf extract respectively while group 5 received 10 mg/kg chloroquine phosphate orally. The treatment continued orally in all the groups daily for 5 days. Geimsa stain thin blood film was prepared from the tail of each mouse on a daily basis to determine parasitemia level. The mean survival time for each group was calculated by finding the average survival time (days) of the mice in each group over a period of 28 days (D0-D27).

The experiment was repeated following the same procedure for the most potent fraction at 50, 100 and 200 mg/kg.

Phytochemical screening

The crude extract and the fractions were screened for the presence of different secondary metabolites using standard methods.[11]

Statistical analysis

Data obtained were expressed as mean ± standard error of mean (SEM). The significance difference between the controls and test groups were determined by one-way analysis of variance (ANOVA), followed by Turkey-Kramer multiple comparism post hoc test. *P*-value less than 5% was considered statistically significant.

RESULTS

Extraction and fractionation yields

The ethanol extract of leaf of *R. smithii* gave 5.31% yield and 30 g of extract yielded 68.05, 9.75, 2.65 and 15.6 % for hexane, ethylaceate, butanol and aqueous fractions respectively.

Acute toxicity

The acute toxicity studies showed that the test samples produced no mortality at the end of the study. No visible sign of toxicity was seen in the behavior of the mice. The test samples were found to be safe up to the dose of 2,000 mg/kg and from these results a 400 mg/kg was chosen for further experimentation as the maximum dose.

Suppressive test

The ethanol leaf extract of *R. smithii* showed dose dependent decrease in the level of parasitemia in mice infected with *P. berghei* parasite. The extract at 100, 200 and 400 mg/kg produced 33.24, 57.51 and 72.79 % suppression respectively. The decrease was statistically significant ($p < 0.05$) at all doses compared to DMSO group but the suppressive activity was however less than that of standard drug chloroquine (Table 1). The extract also significantly increased the survival time at all doses relative to negative control but the effect was significantly ($p < 0.05$) less than that of chloroquine (Table 1). In the fractions, parasitemia was reduced compared to control group however the effect was less than that of chloroquine (Table 2). The order of suppression of the fractions (200 mg/kg) was ethylacetate (84.39%) > butanol (61.41%) > (hexane (57.08%) > aqueous (39.88%). The survival time was significantly ($p < 0.05$) prolonged by ethylacetate and butanol fractions (each 200 mg/kg) compared to negative control group (Table 2).

Table 1: Suppressive effect of ethanol leaf extract of *R. smithii* against *P. berghei* in mice

Drug	Dose (mg/kg)	Mean parasitemia (D5)	% Suppression	Survival date
1% DMSO	0.2 ml	14.59 ± 0.39	-	6.60 ± 0.45
R. smithii	100	9.74 ± 0.08	33.24*	9.78 ± 0.34*
	200	6.20 ± 0.15	57.51*	13.08 ± 0.12*
	400	3.97 ± 0.01	72.79*	16.15 ± 0.67*
Chloroquine	10	0	100*	28.00 ± 0.00*

Values are expressed in mean ± SEM; * indicates significant difference from control at $p < 0.05$; n = 5.

Table 2: Suppressive effect of fractions of ethanol leaf extract of *R. smithii* against *P. berghei* in mice

Drug	Dose (mg/kg)	Mean parasitemia	% Suppression	Survival date
1% DMSO	0.2 mL	13.84 ± 0.33	-	6.28 ± 0.68
Hexane	50	9.24 ± 0.11	33.23*	7.99 ± 0.19
	100	7.10 ± 0.20	48.70*	8.47 ± 0.78*
	200	5.94 ± 0.19	57.08*	9.79 ± 0.67*
Ethylacetate	50	6.48 ± 0.76	53.18*	13.66 ± 0.35*
	100	5.47 ± 0.52	60.48*	15.78 ± 0.45*
	200	2.16 ± 0.11	84.39*	16..67 ±0.78*
Butanol	50	8.04 ± 0.67	41.91*	8.47 ± 0.23*
	100	6.81 ± 0.45	50.81*	12.17 ± 0.12*
	200	5.34 ± 0.15	61.41*	14.64 ± 0.43*
Aqueous	50	10.87 ± 0.99	21.45*	6.89 ± 0.47*
	100	9.86 ± 0.14	28.76*	7.01 ± 0.34*
	200	8.32 ± 0.26	39.88*	8.23 ± 0.67*
Chloroquine	10	0.47 ± 0.01	96.43*	28.00 ± 0.00*

Values are expressed in mean ± SEM; * indicates significant difference from control at $p < 0.05$; n = 5.

3.3 Curative test

There was a progressive dose and time-dependent reduction in parasitemia in the test groups that received extract compared to the vehicle (Table 3). The reduction in parasitemia by chloroquine was significantly ($p < 0.05$) higher that of extract at the tested doses. The survival time was significantly ($p < 0.05$) increased by the extract but less than that of chloroquine (Table 3). The ethylacetate fraction gave the best antimalarial activity among the fractions in 4-day suppressive test and was further tested for its activity on established parasite infection. The fraction demonstrated significant $p < 0.05$) dose dependent reduction of parasitemia compared to the control (Table 3). At 200 mg/kg the fraction produced very good inhibitory effect (71.68%) but chloroquine cleared the parasite to undetectable level on day five and the reduction in parasite level was significantly higher ($p < 0.05$) compared to the vehicle and tested doses of fraction. At 50, 100 and 200 mg/kg ethylacetate fraction significantly increased the survival time compared to the vehicle but the activity was lower than that of chloroquine (Table 3).

Table 3: Parasitaemia and survival of infected mice treated with crude extract and ethylacetate fraction of *R. smithii* in curative test.

Drug	Dose (mg/kg)	Day 1	Day 2	Day 3	Day 4	Day 5	% Inhibition	Survival Date
1% DMSO	0.2 mL	12.64 ± 0.11	16.06 ± 0.39	18.41 ± 1.78	20.20 ± 1.70	22.88 ± 0.12	-	7.03 ± 0.32
R. smithii	50	11.45 ± 0.11	12.16 ± 0.37	11.34 ± 0.24	11.03 ± 0.56	10.83 ± 0.11	52.85*	10.93 ± 0.11*
	100	12.62 ± 0.19	14.69 ± 0.28	12.48 ± 0.31	11.98 ± 0.27	9.93 ± 0.28	56.60*	12.34 ± 0.45*
	200	13.05 ± 0.33	14.28 ± 0.77	12.85 ± 0.30	9.32 ± 0.50	7.98 ± 0.20	65.12*	14.24 ± 0.12*
Ethylacetate fraction	50	13.44 ± 0.41	13.89 ± 0.34	12.34 ± 0.12	10.83 ± 0.45	9.98 ± 0.66	56.38*	12.69 ± 0.34*
	100	12.67 ± 0.45	13.74 ± 0.61	10.45 ± 0.33	9.43 ± 0.73	7.24 ± 0.18	68.36*	14.34 ± 0.78*
	200	13.04 ± 0.15	14.85 ± 0.91	9.48 ± 0.78	7.25 ± 0.23	6.48 ± 024	71.68*	18.23 ± 0.56*
Chloroquine	10	12.57 ± 0.21	8.27 ± 0.29	2.61 ± 0.2	0.56 ± 0.14	0	100*	28.00 ± 0.00*

Values are expressed in mean ± SEM; * indicates significant difference from control at $p < 0.05$; n = 5

3.4 Phytochemical tests

The phytochemical screening of the ethanol extract of *Rutideasmithii*leaf revealed the presence of alkaloids, saponins, terpenoids and phenolic compounds. Cardiac glycosides, anthraquinones and cyanogenetic glycosides were not detected. The phytochemical constituents of the fractions are shown in Table 4.

Table 4: Phytochemical screening of *R. smithii* crude extract and fractions

Secondary metabolites	Crude Extract	Hexane fraction	Ethylacetate fraction	Butanol fraction	Aqueous fraction
Alkaloids	+	-	+	-	-
Anthraquinones	-	-	-	-	-
Tannins	+	-	+	+	+
Flavonoids	+	_	+	+	+
Saponins	+	-	+	+	+
Terpenoids	+	+	+	-	-
Cardiac glycosides	-	-	-	-	-
Cyanogenetic glycoside	-	-	-	-	-

+ detected; -not detected

DISCUSSION

In Nigeria and other developing countries where malaria is endemic, medicinal plants provide easily accessible alternatives method of treatment to widely used antimalarials drugs like quinine and artemisinin that were obtained from medicinal plants; *Cinchona succirubra* and *Artemisia annua* respectively.[12] In this study, *Plasmodium berghei* ANKA strain was used to predict outcomes of suspected antimalarial agent in mice due to its sensitivity to chloroquine and was reported to give the same results like human malaria.[13] Also, previous studies indicated that many synthetic drugs such chloroquine and artemisinin were evaluated for antimalarial potential using the animal model.[14,15]

In toxicity study, the absence of death observed in mice following oral administration of *R. smithii* test samples at 2000 mg/kg suggested that the extract and fractions were safe at the tested doses. Also, the highest dose of extract and fractions used to treat parasite infected mice which elicited antimalarial activity did not cause death in the mice and was much lower than the highest acute dose thus can be considered to be safe. In the antimalarial study, the extract and fractions produced reduction in parasite in mice infected with *P. berghei* indicating that the plant has antimalarial activity. This agrees with previous report that showed antimalarial properties of plants from Rubiaceae family.[16] In the curative study, the crude extract produced progressive reduction in parasitemia after the second dose but the positive control chloroquine started its activity immediately after the first dose. This suggested that the extract might have a delayed-on set of action and that loading dose might be necessary. The level of clearance of parasitemia was seen to increase as the number of days after treatment increases suggesting that extract has long duration of action. Among the fractions, ethylacetate was found to possess highest blood schizontocidal activity as indicated in suppressive results thus suggesting compounds responsible for the antimalarial activity reside mainly in the fraction. The fraction demonstrated dose dependent curative ability with the best effect at the highest dose and also prolonged the mean survival time in established malaria parasite infection. This indicated that the fraction has therapeutic efficacy against established infection. Preliminary phytochemical screening of the leaf extract of *R. smithii* showed the presence of some secondary metabolites like saponins, alkaloids, phenolic and terpenoid compounds. Some these have been shown to have antimalarial activity.[17,18] A number of alkaloids like quinine are known to possess antimalarial activity by inhibiting formation of protein in the parasite[19] thus they might be involved in observed activity. Antimalarial activity of phenolic compounds is due to their ability to intercalate with parasite nucleic acid.[20] These metabolites also act as free radical scavengers that impair oxidative damage induced by malaria parasites.[21] This could be one of mechanisms of action of the extract as it contains phenolics with antioxidant activity. Terpenoid compounds present in the extract are also known to have promising antimalarial property.[22] The fractions from extract demonstrated different degrees of antimalarial activity. This may be due to varying phytochemical constituents found in them. However, compounds found in crude extract and ethylacetate fraction were the same but the ethylcetate fraction showed better activity possibly due to the presence of higher numbers of secondary metabolites that are active against malaria parasites. The suppressive antimalarial activity of aqueous fraction was less than that of ethylacetate fraction indicating difference in the type and concentration of secondary metabolites in the fractions. The phytochemical constituents in the crude extract and fractions could be acting singly or in synergy with one another to produce the observed antimalarial activity. The curative effect of ethylacetate fraction occurred in dose dependent manner and prolonged the mean survival time in established parasite infection. This suggests that the fraction has therapeutic efficacy against established malaria parasite. This property is additive to the suppressive activity thus the ethylacetate fraction of the extract can thus be said to a good source of antimalarial agents since both activities are necessary in a potential phytodrug.[23] However, further work could be carried out in order to isolate and identify the active compounds from the plant responsible for the observed antimalarial activity.

CONCLUSION

The results of this study showed that the ethanol leaf extract of *R. smithii* has significant *in vivo* antimalarial activity against chloroquine sensitive strain of *P. falciparum* that resides mainly in ethylacetate fraction. These findings justify its ethno botanical usage in the treatment of malaria

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