European Journal of Medicinal Plants

24(4): 1-7, 2018; Article no.EJMP.42874
ISSN: 2231-0894, NLM ID: 101583475

In vivo Anti-plasmodial Activity of Crude Extracts of Three Medicinal Plants Used Traditionally for Malaria Treatment in Kenya

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RAO, RSON, HMM and MJM designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Authors JM, HB and SK managed the analyses of the study. Author RAO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2018/42874
Editor(s):
(1) Thomas Efferth, Editor-in-Chief, Phytomedicine, Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Germany.
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(3) Elvis Ofori Ameyaw, University of Cape Coast, Ghana.
Complete Peer review History: http://www.sciencedomain.org/review-history/25755

Original Research Article

Received 13th May 2018
Accepted 21st July 2018
Published 1st August 2018

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ABSTRACT

The aim of the study was to determine the *in vivo* anti-plasmodial activity of three plants *Rhamnus prinoides*, *Rubus keniensis* and *Garcinia buchananii* which are used for malaria treatment by indigenous communities in Kenya. This work was done at the Department of Biological and Preclinical studies, Institute of Traditional Medicine, Muhimbili University of Health & Allied Sciences in October 2016 to August 2017. Male and female albino mice were infected with *Plasmodium berghei* (*ANKA*) in the Peter's four day suppression test. Five groups of mice; Group 1 (solvent: 5 mL/kg body weight of 1% carboxymethyl cellulose), Group 5 (10 mg/kg body weight chloroquine), Groups 2, 3 and 4 were given 200, 400 and 800 mg/kg body weight of plant extracts. The results showed that 5% aqueous methanol extracts of *R*. *prinoides*, *G*. *buchananii* and *R*. *keniensis* exhibited higher anti-plasmodial activity than the 1:1 dichloromethane: methanol extracts in the preliminary testing. The doses showing 50% parasite suppression (EC_{50}) were 139.2, 169.4 and 245.1 mg/kg body weight for *R*. *prinoides*, *G*. *buchananii* and *R*. *keniensis*, respectively. *In vivo* anti-plasmodial activity of the three plants has supported the traditional use of extracts of *Rhamnus prinoides*, *Rubus keniensis* and *Garcinia buchananii* for treatment of malaria. Isolation of compounds from these plants is in progress.

Keywords: Anti-plasmodial; in vivo activity; Rhamnus prinoides; Garcinia buchananii; Rubus keniensis.

1. INTRODUCTION

Malaria is a life-threatening disease caused by protozoa of the Genus *Plasmodium*. It is transmitted through the bites of infected female anopheles mosquito. In 2016, 216 million malaria cases were reported in 91 countries, an increase of 5 million cases over 2015. Malaria deaths reported in 2016 were 445,000 globally with Africa accounting for 90% malaria cases and 91% deaths. Malaria remains a major cause of morbidity and mortality in sub-Saharan Africa [1]. Malaria control has had a challenge due to lack of access to effective malaria control methods, resistance to available antimalarial drugs and insecticides [2]. Resistance to Artemisinin combination therapies (ACTs) has been reported in South East Asia [3,4] and Equatorial Guinea [5]. Additionally, resistance to other alternative drugs such as mefloquine, amodiaquine, sulphadoxine-pyrimethamine has been reported in most malaria endemic areas [6]. Therefore, there is a need to intensify efforts to search for new, safe and affordable anti-malarial compounds with unique mechanisms of action. Medicinal plants have played an important role in the development of anti-malarial drugs that are currently in use [7] and still provide a rich niche for the discovery of new compounds that have an anti-malarial activity or which may be used as templates to develop new anti-malarial drugs. Thus, we evaluated the anti-malarial activity of *Rhamnus prinoides*, *Rubus keniensis* and *Garcinia buchananii* that are used in East African folklore for the treatment of malaria infection.

2. MATERIALS AND METHODS

2.1 Chemicals

Dichloromethane and methanol were obtained from Kobian Limited Nairobi, Kenya. Diethyl ether, methanol, and Giemsa stains were purchased from Techno-Net Scientific (Dar es Salaam, Tanzania). Chloroquine diphosphate was bought from Sigma (Sigma®, Stenheim, Germany).

2.2 Malaria Parasites

Chloroquine sensitive *Plasmodium berghei* (*ANKA* strain) were donated by Dr. Lindsay Stewart of the Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, United Kingdom. The parasites are preserved by sequential passage of infected blood to non-infected mice once every week.

2.3 Collection and Extraction of Plant Material

The plants were collected with the aid of a botanist in May, 2015 and voucher specimens: - *Rubus keniensis* (RO2015/02), *Rhamnus prinoides* (RO2015/03) and *Garcinia buchananii* (RO2015/05) are deposited in the Herbarium at the Department of Botany, University of Nairobi. *Rubus keniensis* belongs to the family *Rosaceae*, *Rhamnus prinoides* is found in the family *Rhamnaceae* while *Garcinia*

buchananii belongs to the family *Clausiaceaem*. The plant material was air dried under shade for a period of 14 days and ground into powder. Solvent extraction was done by maceration using dichloromethane: methanol (1:1) and 5% aqueous methanol. The solvents were removed under reduced pressure using a rotary evaporator to obtain crude extracts. The extracts were stored at -20º C until the time of use. The percentage yield are recorded in Table 1.

$$\% \text{ yield} = \frac{weight\ of\ the\ crude}{weight\ of\ dried\ sample} \times 100$$

2.4 *In vivo* Anti-malarial Activity Assay

A modified Peter's 4-day suppression test was used to evaluate the anti-plasmodial activity of the plant extracts in a mouse model infected with *Plasmodium berghei ANKA* strain [8]. Animals were treated with 200, 400 and 800 mg/kg body wt of aqueous-methanol extracts.

2.5 Animals

Young adult Theiller's original white albino mice weighing 20-30 g of both sexes were used. The mice were acclimatized to laboratory conditions, given food and water ad libitum for five days before the experiments. The animals were handled according to National and International guidelines for handling of laboratory animals.

2.6 Preparation of Infected Red Blood cells Suspension and Parasite Inoculation

Donor mice with a parasitaemia of 20-30% were used to infect other mice in the 4-day suppression test. The donor mice were anesthetized using diethyl ether and blood removed through cardiac puncture. The blood was diluted by normal saline (0.9% w/v sodium chloride) to make a suspension of 1×10^8 infected red blood cells (iRBCs) per mL. 25 mice were used for each experiment per plant extract. They were weighed and randomized into five groups. 5 mice were used in each treatment group and 5 mice each in both control groups. Each mouse used in the experiment was infected via the tail vein with 0.2 mL of diluted infected blood.

2.7 Administration of the Extracts

The extracts were suspended in 1% carboxymethyl cellulose (CMC). Chloroquine was dissolved in normal saline. After three hours of infection, the mice were randomly distributed into groups of five. The negative control group received 1% CMC (5mL/kg body wt/day), the positive control group was given 10 mg/kg body wt/day chloroquine and the treatment groups received extracts at oral doses of 200, 400, 800 mg/kg body wt/day, respectively. The dosing was done daily, starting with the day of infection and continued for four days. Body weight was measured daily and parasitaemia determined at the end of the fourth day. In the pre-run 5% aqueous methanol and 1:1 DCM: MeOH of the three plants were tested at a dose of 400 mg/kg body wt to determine the one giving higher parasite suppression. The 5% aqueous methanol extract of *R*. *prinoides*, *G*. *buchananii* and *R*. *keniensis* gave higher parasite suppression (61.4 vs 60.1% and 57.4 vs 24.5%, 42.4 vs 38.4%), respectively. Extracts with high % parasite suppression were then used for dose-response studies.

2.8 Determination of % Parasitaemia and Suppression

On the fourth day, blood was collected from tail veins of the mice, smeared onto a microscope slide and fixed with absolute methanol. The slides were stained with 10% Giemsa in phosphate buffer (pH 7.2) for 20 minutes. The parasites were counted under a microscope at ×100 oil immersion. Images of the parasites captured by a camera fitted on a microscope (Tension®, Axiom) and processed by Tuscen TS view version 6.2.3.3 (Tuscen, 2013) were also used to support counting of the parasites.

Percentage parasitaemia was determined by counting infected red blood cells in at least 1000 total red blood cells [9]. Kalra *et al* protocol was used to calculate percentage parasitaemia [10]

$$\% \text{ parasitaemia} = \frac{Number\ of\ infected\ erythrocytes}{Total\ number\ of\ erythrocytes} \times 100$$

The percentage suppression was calculated as follows:-

$$\frac{Mean\ \%\ parasitaemia\ in\ negative\ control\ mice - mean\ \%\ parasitaemia\ in\ treated\ mice}{Mean\ \%\ parasitaemia\ in\ negative\ control\ mice} \times 100$$

Table 1. Percentage yield of the plant extracts

Plant	Dry sample (kg)	DCM: MeOH (1:1) (g)	% Yields	5% H_2O/ MeOH (g)	% Yields
G. buchananii (stem bark)	4.3	425.6	9.9	185.18	4.3
R. keniensis (root bark)	2.1	221.09	10.5	58.46	2.8
R. prinoides (root bark)	1.4	155.14	11.1	46.14	3.3

2.9 Statistical Analysis

The results are presented as mean % parasitaemia and mean survival time (mean ± SEM). Data was analyzed using SPSS version 20. The means were compared by One Way ANOVA followed by post-hoc Tukey's test to compare results between treatment groups and control groups. Differences were considered statistically significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSIONS

The extract of *Rhamnus prinoides*, *Garcinia buchananii* and *Rubus keniensis* exhibited good to moderate anti-plasmodial activity with 50% parasite suppression doses of 139.2, 169.4 and 245.1 mg/kg body wt, respectively. The results in Tables 2-4 show that the 5% aqueous methanol extracts of *R. prinoides*, *G. buchananii* and *R. keniensis* at doses of 200, 400 and 800 mg/kg body wt caused dose-dependent parasite growth suppression. In the case of *Rhamnus prinoides* and *Garcinia buchananii* there were significant differences in parasite suppression between the three doses used and between each of the three doses used and the solvent control groups ($P \leq 0.05$). Similarly, the extract of *Rubus kenienis* exhibited dose-dependent suppression of parasite growth with significant differences between each of the three doses as compared to the solvent control group ($P \leq 0.05$). The only

Table 2. *In vivo* activity and mean survival time for *Rhamnus prinoides*

Treatment	Mean % parasitaemia at day 4 ± SEM	Mean survival time (days)	% suppression
Group 1: 1% CMC	24.14 ± 0.77	8.2±0.58	0
Group 2: 200 mg/kg body wt/day	13.32 ± 0.24*[a,c,d]	13.4±0.93	44.82
Group 3: 400 mg/kg body wt/day	10.2 ± 0.39*[a,b,d]	16.4±1.44*[a]	57.74
Group 4: 800 mg/kg body wt/day	8.12 ± 0.40*[a,b,c]	19.0±1.78*[a]	66.36
Group 5: CQ10 mg/kg body wt/day	0.00	27.0±1.08	100

*The results are presented as mean ± SEM (n=5) * significant difference at P ≤ 0.05). Key: CQ- chloroquine, [a] compared to negative control; [b] compared to 200 mg/kg body wt; [c] compared to 400 mg/kg body wt; [d] compared to 800 mg/kg body wt*

Table 3. *In vivo* activity and mean survival time for *Garcinia buchananii*

Treatment	Mean % parasitaemia at day 4 ± SEM	Mean survival time (days)	% suppression
Group 1: 1% CMC	21.42 ± 0.94	6.4±0.75	0
Group 2: 200 mg/kg body wt/day	13.22 ± 1.63*[a,d]	8.0±0.58*[a]	38.28
Group 3: 400 mg/kg body wt/day	9.72 ± 0.43*[a]	13.0±0.91*[a]	54.62
Group 4: 800 mg/kg body wt/day	7.60 ± 0.62*[a,b,c]	15.8±1.70*[a]	64.51
Group 5: 10 mg/kg body wt/day	0.00	23.3±1.75	100

*The results are presented as mean ± SEM (n=5) * significant difference at P ≤ 0.05). Key: CQ- chloroquine, [a] compared to negative control; [b] compared to 200 mg/kg body wt; [c] compared to 400 mg/kg body wt; [d] compared to 800 mg/kg body wt*

Table 4. *In vivo* activity and mean survival time for *Rubus keniensis*

Treatment	Mean % parasitaemia at day 4 ± SEM	Mean survival time (days)	% suppression
Group 1: 1% CMC	27.92 ± 0.66	6.0±0.55	0
Group 2: 200 mg/kg body wt/day	22.04 ± 1.05*[a,d]	7.25±0.85	21.06
Group 3: 400 mg/kg body wt/day	15.60 ± 1.19*[a,d]	11.2±0.74	44.12
Group 4: 800 mg/kg body wt/day	12.04 ± 0.49*[a,b,c]	16.0±1.47*[a]	56.88
Group 5: CQ 10 mg/kg body wt/day	0.00	24.3±1.93	100

*The results are presented as mean ± SEM (n = 5) * significant difference at P ≤ 0.05). Key: CQ- chloroquine, [a] compared to negative control; [b] compared to 200 mg/kg body wt; [c] compared to 400 mg/kg body wt; [d] compared to 800 mg/kg body wt*

significant difference between the doses was at 800 mg/kg body wt as compared to the lower doses (P≤ 0.05). Chloroquine given at 10mg/kg body wt caused 100% suppression of the parasites. The survival times at the three doses for each of the three plants were all higher compared to the negative control groups, although not all doses showed statistically significant differences, especially for *R. keniensis* extracts.

In vivo anti-plasmodial activity can be grouped as moderate, good and very good if an extract showed a respective % parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg body wt body weight daily [11]. According to this classification, the extracts of *R. prinoides* and *G. buchananii* showed good activity while that of *R. keniensis* showed a moderate activity. The results of the *in vivo* four-day suppression test also indicate that the plants showed dose-dependent suppression of growth of the parasites in mice. The 5% aqueous methanol extract of *R. prinoides* showed the highest activity with % suppression of 66.4 at 800 mg/kg body wt while the extract of *R. keniensis* showed the least activity with % suppression of 56.9 at 800 mg/kg body wt. The root bark chloroform extract of *R. prinoides* was previously reported to exhibit in vitro anti-plasmodial activity with IC_{50} of 3.50 against the D6 *Plasmodium falciparum* strain [12]. In addition, the aqueous extract of this plant has been previously tested for *in vivo* anti-malarial activity and it exhibited a % suppression of 51.3% at 500 mg/kg body wt body weight [13]. The anti-malarial activity of *R. prinoides* reported in this paper reinforces the biological evidence of use of this plant in the treatment of malaria. Emodin and emodinanthrone rhamnoside acetate have been isolated from this plant [14]. Emodin tested against CQ sensitive (Pf3D7) and CQ resistant (Pfk1) strains of *P. falciparum* gave an IC_{50} of 16. 2 µM and 37 µM, respectively [15].

According to our findings, *R. keniensis* also showed dose-dependent parasite growth suppression in mice (Table 4). This is the first time the *in vivo* anti-plasmodial activity of *R. keniensis* is being reported, and literature search shows that no bioactive compounds have been isolated from the plant. This plant is used to treat stomach ache, sexually transmitted infections and malaria among some communities living in Kenya [16,17]. The methanol extract of the root bark showed good antibacterial activity against *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumonia* and *Staphylococcus aureus*. Phytochemical screening indicated the presence of flavonoids, anthraquinones, saponins, steroids, reducing sugars and polyoses [18].

The aqueous extract of *G. buchananii* has been reported to have anti-diarrheal and anti-oxidant activity [19,20] but there is no previous study that has reported its anti-plasmodial activity. Biflavanones 1a-1d have been reported from this plant and these compounds have been found to have anti-oxidant activity [21]. Biflavanoids isolated from other species of the same genus have been reported to exhibit *in vivo* anti-malarial activity. Biflavanones isolated from *Garcinia livingstonei* have shown an *in vivo* suppression of the malaria parasites ranging from 39.3 – 61% at doses of 25, 50, 100 and 200 mg/kg body wt [21]. Additionally, kolavorin 1 and 2 isolated from *Garcinia kola* showed a suppression of *P. berghei* in Swiss albino mice of 85% and 90%,

respectively at a dose of 100 mg/kg body wt given daily for four days [22].

4. CONCLUSION

Through *in vivo* anti-plasmodial testing, evidence has been established to support the traditional use of extracts of *Rhamnus prinoides*, *Rubus keniensis* and *Garcinia buchananii* for treatment of malaria. Research is ongoing to isolate compounds from these plants. We do recommend that acute and chronic toxicity test to be done on this extracts to determine their safety in animal models.

CONSENT

It is not applicable

ETHICAL APPROVAL

The study was given ethical clearance by the MUHAS institutional review board (IRB) (reference no. 2015-09-25/aec/vol.x/03).

ACKNOWLEDGEMENT

Appreciation to German Academic Exchange Programme (DAAD) for funding this PhD work. Much thanks to Patrick Mutiso for identifying the plants. We appreciate Nicodemus Michael for his technical support in the *in vivo* screening.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/25755