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ORIGINAL PAPER

In vitro anti-plasmodial and in vivo anti-malarial activity of some plants traditionally used for the treatment of malaria by the Meru community in Kenya

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Abstract Extracts of seven medicinal plant species used for treatment of malaria in traditional/cultural health systems of the Ameru people in Kenya were tested in vitro and in vivo against *Plasmodium falciparum* (D6 and W2 strains) and *P. berghei*, respectively. Of the plants tested, 28.57% were highly active (IC_{50} <10 μg/ml) and 42.86% moderately active (IC_{50} 10–50 μg/ml), while 28.57% had weak activity of 50–125 μg/ml in vitro. The water and methanol extracts of *Boscia salicifolia* Oliv. and *Artemisia afra* Jacq. (ex-Willd.) were the most active against both the chloroquine (CQ)-sensitive (D6) and the CQ-resistant (W2) *P. falciparum* strains. *Artemisia afra* and *Rhus natalensis* Bernh. (ex-Krauss) exhibited the highest parasite clearance and chemo-suppression (>70%) in vivo (in mice). The plants with high in vitro anti-plasmodial (low IC_{50} values) and high anti-malarial activity (high chemo-suppression) in vivo are potential sources of novel anti-malarial drugs.

Keywords Anti-malarial · Anti-plasmodial · Toxicity · *Boscia salicifolia* · *Artemisia afra* · *Rhus natalensis*

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Introduction

Malaria is one of the most important infectious diseases in the world, especially the tropics [1]. It is estimated to cause 2.3 and 9% of the disease burden in the world and Africa, respectively [2], thus ranking third after pneumococcal acute respiratory infections (3.5%) and tuberculosis (TB) (2.8%). The incidence of malaria is increasing, with an estimated 300–500 million cases and 3 million deaths annually [3]. Malaria mortality and morbidity are increasing in the highest risk group, which is children under 5 years of age in Africa [4], due to the increasing resistance of the parasites to the available drugs [5]. Drug resistance by *P. falciparum*, the species that causes the greatest number of malaria cases and deaths in Africa [6], has become an issue of utmost concern [7–8] and was implicated in enhanced mortality in hyper- and holoendemic areas and the development of new and expanding foci of *falciparum* malaria in the last 5 decades [9]. It has been identified as a major factor in the economic constraints in malaria control [10]. Consequently, there is an urgent need to discover, develop and use new anti-malarial drugs.

Plant-derived drugs offer another window in chemotherapy [11–12]. Some of the anti-malarial drugs in use today were obtained directly from plants or developed using plant-derived chemical structures as templates. For

example, quinine was developed from *Cinchona* sp. [13], and cheap analogues like chloroquine, mefloquine and amodiaquine were developed using the parent molecule as a template. Relatively simple chemical modifications of artemisinin have led to a series of potent anti-malarial drugs (artemether, arteether and artesunate) that are playing an increasingly important role in the treatment of malaria [14]. In Kenya, plant extracts are still widely used in the treatment of malaria and other ailments [15]. A large number of local plants have been studied to establish their potential in malaria treatment [16–20]. Only about 20% of the plants with acclaimed medicinal values have been subjected to rigorous scientific screening and evaluation [21]. In continuation of the efforts to verify the efficacy of traditional/cultural/folkloric anti-malarial preparations, we subjected seven species of medicinal plants to in vitro antiplasmodial and in vivo anti-malarial (rodent) screening. The results are presented in this paper.

Materials and methods

Collection, processing and extraction of plant materials

Following a survey on the ethno-medical use of local plants in the Meru region in the Eastern Province of Kenya through interviews with traditional health practitioners (THPs), seven were selected (Table 1) for the study. The plants were botanically authenticated by a taxonomist and voucher specimens deposited at the East African Herbarium, National Museums of Kenya (NMK). Several plant parts including the leaves, roots, stem or stem-barks depending on the part used by the THPs were collected in August 2004. The plant parts were air-dried under shade for 14 days, then the root, stems (twigs), stem-bark or root-bark were ground using a laboratory mill (Christy and Norris Ltd., Chelmsford, England) while a kitchen blender was used to grind the leaves. Ground plant material (100 g) was extracted with sterile distilled water. Similarly, 100 g of the plant material was separately extracted in methanol. The water extracts were lyophilized in a freeze-dryer while the methanol extracts were concentrated using a rotary evaporator. The extract was weighed and expressed as a percentage yield (Table 1).

Bioassays

Stock solutions

Stock solutions for in vitro assay of plant extracts were made in deionized autoclaved water and re-sterilized by passing through 0.22-μm micro-filters in a laminar flow hood. The water-insoluble extracts were dissolved in 100% dimethylsulfoxide (DMSO) (Sigma Chemical Co., St Louis, MO) followed by a subsequent dilution to lower concentration of DMSO to ≤1% to avoid solvent carry over (solvent) effect [22]. Stock solutions (1 μg/ml) of chloroquine diphosphate, artemisinin and mefloquine were similarly prepared for use as reference drugs. All the drug solutions were stored at –20°C until required for bioassay.

Parasite cultures

Test samples were screened against two strains of *P. falciparum*, Sierra Leonean D6 (CQ-sensitive) and Indo-Chinese W2 (CQ-resistant). The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Nairobi. The parasites were

Table 1 Yield (%) of solvent extracts of selected medicinal plants from Meru

Plant	Specimen number	Family	Part	Extract	Yield (%)
Artemisia afra Jacq.	CM035	Asteraceae	Leaves	H_2O	8.6
				MeOH	2.0
Boscia salicifolia Oliv.	CM039	Capparidaceae	Stem bark	H_2O	14.0
				MeOH	6.4
Catharanthus roseus (L.) Don	CM030	Apocynaceae	Leaves	H_2O	13.9
				MeOH	4.0
Clutia robusta Pax, E, FZ.	CM031	Euphorbiaceae	Leaves	H_2O	10.3
				MeOH	2.3
Cyathula schimperiana Igifashi	CM043	Amaranthaceae	Root	H_2O	8.5
				MeOH	2.6
Rhus natalensis Bernh.	CM037	Anacardiaceae	Stem bark	H_2O	10.0
				MeOH	1.9
Ximenia americana L.	CM033	Olacaceae	Root bark	H_2O	8.0
				MeOH	2.8

cultivated by a previously described in vitro technique [23]. The culture medium consisted of RPMI 1640 supplemented with 10% human serum [24]. Uninfected human blood group O Rh-positive erythrocytes (<28 days old) served as host cells. The cultures were incubated at 37°C in an atmosphere of 3% CO_2, 5% O_2 and 92% N_2 (BOC, Nairobi).

In vitro anti-plasmodial assay

An in vitro semi-automated micro-dilution assay technique that measures the ability of the extracts to inhibit the incorporation of [G-^{3}H]hypoxanthine (Amersham International, Burkinghamshire, UK) into the malaria parasite was adopted [25]. Briefly, aliquots of the culture medium (25 µl) were added to all the wells of a 96-well flat-bottomed micro-culture plate (Falcon, Becton Dickinson, Franklin Lakes, NJ). Aliquots of the test solutions (25 µl) were added in duplicate to the first wells, and a Titertek motorized hand diluter (Flow laboratories, Uxbridge, UK) was used to make serial twofold dilutions of each sample over a 64-fold concentration range. Negative controls treated with solvent (DMSO) alone were also added to each set of experiments [26] to check for any solvent effect. The susceptibility tests were carried out with initial parasitemia (expressed as the percentage of erythrocytes infected) of 0.4% by applying 200 µl, 1.5% hematocrit, *P. falciparum* culture to each well. Two hundred microliters of culture media without parasites was added into four wells on the last row of each plate to serve as a background. Parasitized and non-parasitized erythrocytes were incubated at 37°C in a gas mixture, 3% CO_2, 5% O_2 and 92% N_2 for 48 h after which 25 µl of culture medium containing 0.5 μCi of [G-^{3}H]hypoxanthine was added to each well. The culture plates were further incubated for 18 h. At the end of the incubation period, the radio-labeled cultures were harvested onto glass-fiber filters using a 96-well cell harvester. [G-^{3}H]hypoxanthine uptake was determined using a microbeta trilux liquid scintillation and luminescence counter (Wallac MicroBeta TriLux). The drug concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (cpm) using the formula:

$$IC_{50} = \text{antilog}\,(\log X_1 + [(\log Y_{50} - \log Y_1) \times (\log X_2 - \log X_1)]/(\log Y_2 - \log Y_1))$$

where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1,Y_1, X_2 and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints [27].

In vivo anti-malarial assay

Male Swiss mice (6–8 weeks old, weighing 20 ± 2 g) infected with *P. berghei* ANKA strain were used for anti-malarial assay. The parasite strain was maintained by serial passage of infected blood by interperitonial (i.p.) injection. The assay protocol was based on the 4-day suppressive test [28]. Briefly, an aliquot of 0.2 ml (2×10^7 parasitized erythrocytes) from an infected donor mouse was transfused into uninfected experimental mice through i.p. injection [29–30]. The infected mice were randomly divided into groups of five individuals each for every test sample. The test extracts were prepared by dissolving the aqueous extracts in sterilized water. Methanol extracts were dissolved in 10% w/v tween 80. CQ diphosphate dissolved in sterilized water was used as a reference anti-malarial drug.

Post-infection (2–4 h on day 0) experimental mice groups were treated with a single dose of test sample at 100 mg/kg at a volume of 0.02 ml by i.p. injection. On days 1 to 3 (24, 48 and 72 h post-infection), the experimental groups of mice were treated again with the same dose and by the same route as on day 0. Two goups (5 mice each) served as negative and positive controls, respectively. The negative group received a placebo (saline) while the positive one was treated with 5 mg/kg day of the reference drug (CQ diphospate).

Parasitemia was determined daily (24-h interval) by a thin blood film sampled from the tail and stained in 10% Giemsa solution. A thin blood smear was similarly made on day 4 (24 h after the last treatment or 96 h post-infection) from all the animals and stained as described above. Parasitemia was calculated by counting four fields of approximately 200 erythrocytes per field under a microscope. The difference between the mean value of the control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent parasitemia suppression (PS) (activity) according to the following formula [31]:

$$PS = \{(A - B)/A\} \times 100$$

where A is the mean parasitemia in the negative control group on day 4 and B the corresponding parasitemia in the test group. The standard deviations for the mean values were calculated as previously described [32]. For all the groups of experimental mice used, the survival time (in days) was recorded and the mean for each group calculated.

Cytotoxicity evaluation

An actively dividing sub-confluent Vero cell growth-inhibition assay was done [33]. Briefly, Vero cells were grown

in Eagle's minimum essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) in 25-ml cell-culture flasks incubated at 37°C in 5% CO_2. Upon attainment of confluence, the cells were seeded at a concentration of 5×10^4 cells/well in 24-well plates and incubated at 37°C for 2 days. The culture medium was replaced by fresh medium containing test extracts at different concentrations and the cells incubated further for 2 days. Cells in triplicate wells for each sample were detached by trypsinization, and the number of viable cells determined by a tryphan blue exclusion test. A hemocytometer was used to aid in counting viable cells. Inhibition data were plotted as dose-response curves, from which the 50% cell growth inhibitory concentration (CC_{50}) was estimated. The selectivity index (SI) was used as the parameter of clinical significance of the test samples by comparing general toxins and the selective inhibitory effect on *P. falciparum*, calculated as: CC_{50} (vero)/IC_{50} (*P. falciparum*) [34].

Results

Activity criteria in the in vitro assay were defined as high when IC_{50} was below 10, moderate when between 10 and 50, and low when between 50 and 100 μg/ml. Samples with IC_{50} >100 μg/ml were considered as inactive. The anti-plasmodial activity of the seven medicinal plants against CQ sensitive/mefloquine-resistant *P. falciparum* (D6 strain) and CQ resistant/mefloquine-sensitive (W2 strain) are summarized in Table 2.

Extracts of *B. salicifolia* were the most active (IC_{50} range 1.04 ± 0.03 to 10.09 ± 1.56 μg/ml) against both *P. falciparum* strains followed by that of *A. afra* (IC_{50} 3.98 ± 0.98 to 11.23 ± 1.98 μg/ml). The methanol extract of *C. roseus* also exhibited high anti-plasmodial activity (4.65 ± 0.08 to 5.34 ± 0.65 μg/ml) against the CQ-sensitive and resistant strains. Apart from *B. salicifolia* and *A. afra*, the water extracts exhibited lower in vitro anti-plasmodial activity than their respective methanol ones in most cases. The water extracts of *X. americana*, *R. natalensis* and *C. schimperiana* were inactive (IC_{50} > 100 μg/ml), while that of *C. roseus* showed moderate activity (IC_{50} 10–50 μg/ml).

Anti-malarial effects of extracts from seven selected medicinal plants on *P. berghei* ANKA in mice are summarized in Table 3. The suppression of parasitemia (chemo-suppression) and mean survival rate (time) were used as measures of efficacy. Samples were categorized as highly active when chemo-suppression was above 60% or moderately active between 30 and 60%, but lowly active below 30%.

Methanol extract of *B. salicifolia* and water extract of *R. natalensis* were the most active with chemosuppression of 86.50 ± 4.37 and 83.15 ± 3.61%, respectively. Both water and methanol extracts of *A. afra* also showed high chemo-suppression (70.25 ± 5.59 and 77.45 ± 3.06%, respectively). The water extracts of *B. salicifolia*, *C. roseus* and *C. robusta* showed moderate chemo-suppression of parasitemia (43.75 ± 6.90, 42.36 ± 4.87 and 42.35 ± 2.15%, respectively). Similarly, the methanol extracts of *R. natalensis, C. schimperiana*, *C. roseus* and *X. americana*

Table 2 In vitro anti-plasmodial activity (IC_{50}) of extracts of selected medicinal plants IC_{50} ± SD (μg/ml)

Plant	Extract	*P. falciparum* (D6)	*P. falciparum* (W2)
Artemisia afra	MeOH	9.04 ± 0.54	3.98 ± 0.98
	H_2O	11.23 ± 1.98	4.65 ± 0.64
Boscia salicifolia	MeOH	1.04 ± 0.03	8.86 ± 0.24
	H_2O	3.65 ± 0.78	10.09 ± 1.56
Catharanthus roseus	MeOH	4.65 ± 0.88	5.34 ± 0.65
	H_2O	32.36 ± 4.31	36.17 ± 7.79
Clutia robusta	MeOH	3.41 ± 0.02	7.58 ± 0.09
	H_2O	71.24 ± 1.69	157.81 ± 5.66
Cyathula schimperiana	MeOH	10.40 ± 1.25	17.61 ± 2.61
	H_2O	175.92 ± 14.45	>250
Rhus natalensis	MeOH	76.84 ± 8.09	98.32 ± 6.48
	H_2O	111.60 ± 22.76	105.25 ± 18.36
Ximenia americana	MeOH	16.74 ± 2.31	68.19 ± 0.12
	H_2O	106.66 ± 29.39	129.23 ± 6.55
Artemisinin		0.00164 ± 0.00011	0.00438 ± 0.00049
CQ diphosphate		0.00311 ± 0.00121	0.04057 ± 0.00052
MQ		0.01608 ± 0.00265	0.00255 ± 0.00003

Table 3 Effect of extracts (100 mg/kg day) of selected medicinal plants on *P. berghei* infected mice

Plant	Extract	Parasitemia (%)	Chemo-suppression (%)	Mean survival time (days)
Artemisia afra	MeOH	8.45 ± 0.27	77.45 ± 3.06	19.21 ± 3.57
	H_2O	11.15 ± 3.01	70.25 ± 5.59	17.85 ± 2.81
Boscia salicifolia	MeOH	5.06 ± 0.84	86.50 ± 4.37	19.08 ± 4.22
	H_2O	21.08 ± 4.52	43.75 ± 6.90	15.71 ± 5.41
Catharanthus roseus	MeOH	24.51 ± 4.32	34.60 ± 1.77	10.85 ± 2.50
	H_2O	21.60 ± 2.51	42.36 ± 4.87	12.39 ± 2.95
Clutia robusta	MeOH	28.81 ± 1.40	23.10 ± 6.43	10.45 ± 3.25
	H_2O	21.60 ± 2.39	42.35 ± 2.15	12.82 ± 4.70
Cyathula schimperiana	MeOH	20.58 ± 1.69	45.08 ± 6.93	15.38 ± 4.18
	H_2O	30.33 ± 2.45	19.06 ± 5.33	7.65 ± 1.89
Rhus natalensis	MeOH	16.40 ± 1.82	56.24 ± 4.85	13.43 ± 5.24
	H_2O	6.31 ± 0.97	83.15 ± 3.61	16.52 ± 1.87
Ximenia americana	MeOH	23.80 ± 7.30	36.49 ± 0.28	7.85 ± 1.59
	H_2O	29.40 ± 3.89	21.55 ± 6.27	6.72 ± 1.52
CQ diphosphate		0.38 ± 0.43%	99.02 ± 1.26%	21.25 ± 5.28
PBS		37.47 ± 6.62%	0.00	7.14 ± 2.14

PBS phosphate buffered saline (negative control), chloroquine diphosphate (standard drug) administered at 5 mg/kg day; $n = 5$

showed moderate activity with 56.24 ± 4.85, 45.08 ± 6.93, 34.60 ± 1.77 and 36.49 ± 0.28% chemo-suppression, respectively. *Ximenia americana* and *C. schimperiana* water extracts and the methanol extract of *C. robusta* exhibited the least chemo-suppression of parasitemia (<30%).

Mean survival time of the experimental groups of test animals correlated to treatment. The survival period of mice that received extracts shown to have a high percent of chemo-suppression lived longer compared to those with low activity or no treatment. Mice treated with the methanol extract of *A. afra* had a mean survival time of 19.21 ± 3.57 days while that of the untreated group was 7.14 ± 2.14 days. These differences were statistically significant in some treatments when compared to the control group using the Dunnett multiple comparison procedure [35–36] (PC-PCSM Software, Version 6.0, 1992, Delta-soft, Meylan, France). These included water and methanol extracts of *A. afra* and *B. salicifolia* ($P < 0.05$). Other tested extracts that prolonged the survival time of mice significantly was the water extract of *R. natalensis* ($P < 0.05$). Where survival time of mice was not significantly different from the untreated control group, deaths may be attributed to the effect of high

Table 4 Cytotoxic effect of selected medicinal plants on Vero cells

Plant	Extract	CC_{50} (μg/ml)	D6 SI	W2 SI
Artemisia afra	MeOH	594.85	65.8	149.46
	H_2O	2,825.21	251.58	607.57
Boscia salicifolia	MeOH	304.92	293.19	34.42
	H_2O	1,683.95	461.36	166.89
Catharanthus roseus	MeOH	167.52	36.03	31.37
	H_2O	1,285.74	39.73	35.55
Clutia robusta	MeOH	460.29	134.98	60.72
	H_2O	4,352.76	61.1	27.5
Cyathula schimperiana	MeOH	241.34	23.21	13.7
	H_2O	2,907.12	16.52	ND
Rhus natalensis	MeOH	211.78	2.76	2.15
	H_2O	3,958.16	35.47	37.7
Ximenia americana	MeOH	198.11	11.83	2.91
	H_2O	1,720.38	16.13	13.31

D6 SI Selectivity index calculated as CC_{50}/IC_{50} tested against *P. falciparum* D6, *W2 SI* selectivity index calculated as CC_{50}/IC_{50} tested against W2, *ND* not done since IC_{50} >250 μg/ml

parasite density or as a result of the toxicity of the extracts.

The highest selective inhibition (ratio of 50% cytotoxic concentration to 50% effective anti-plasmodial concentration) of *P. falciparum* was observed with the water and methanol extracts of *A. afra* and water extract of *B. salicifolia* with selectivity index (SI) ratios of >100 (Table 4). Methanol extract of *C. robusta* also depicted a high selective index. Methanol extract of *R. natalensis* was the most cytotoxic (CC_{50} 211.78 μg/ml, SI 2.15–2.76) followed by *X. americana* and *C. schimperiana* (SI 2.9–23.21). *Catharanthus roseus* was moderately cytotoxic.

Discussions

Herbal medicine is used by up to 80% of the population in the developing countries [37]. Despite the widespread use, few scientific studies have been undertaken to ascertain the efficacy of traditional remedies. The present investigation demonstrates that water and methanol extracts of selected plants are potential candidates for the discovery of effective anti-malarial drugs or lead compounds. *Catharanthus roseus* exhibited high in vitro anti-plasmodial activity, which may be due to the presence of compounds such as alkaloids, terpenoids [38], flavonoids [39] and sesquiterpenes [40] that were previously isolated from the plant. However, the high in vitro anti-plasmodial activity exhibited was lost when the same was subjected to in vivo assay. *Artemisia afra* has previously been shown to exhibit good in vitro activity against the CQ-sensitive (PoW) and CQ-resistant (Dd2) *P. falciparum* strains [41]. Seven flavonoids have so far been isolated from this plant through bioassay-guided fractionation with acacetin, genkwanin and 7-methoxyacacetin exhibiting good in vitro anti-plasmodial activity (IC_{50} 4.3–12.6 μg/ml) [41]. Our study confirms similar levels of anti-plasmodial activity and low toxicity of *A. afra* extracts. The in vivo activity of *A. afra* methanol and water extracts was higher than most of the tested plants. *Artemisia afra* is therefore a good candidate with high potential for the discovery of anti-malarial drugs. *Boscia salicifolia* also had high in vitro anti-plasmodial activity (1.04–10.09 μg/ml) against both the CQ-sensitive (D6) and resistant (W2) *P. falciparum* strains. However, the in vivo activity of *B. salicifolia* water extract was moderate while that of the methanol one was high (43.75 ± 6.90 and 86.50 ± 4.37%, respectively). To our knowledge, this is the first report of the anti-plasmodial activity of *B. salicifolia*. The high in vivo anti-malarial activity of *R. natalensis* is also being reported for first time in this study. The methanol extract of *X. americana* stem-bark was previously screened against *P. falciparum* (W2) and the reported activity (IC_{50} > 50 (g/ml) agrees with our findings [42] and is supported by earlier findings [43]. Mild brine shrimp lethality (LC_{50} 11.25 μg/ml) has also been reported for the same extract [44]. This has been confirmed by Vero cell cytotoxicity assay (Table 4). However, the aqueous extract has much lower toxicity. In this study, in vitro activity for several plants was lost in vivo, and yet others with low in vitro activity demonstrated high in vivo activity. Biotransformation or poor bioavailability of the anti-plasmodial compounds may explain the lack of in vivo anti-malarial activity. Cases of high in vivo, but low in vitro anti-plasmodial activity are common, and several reasons have been advanced. Previous studies have shown that, owing to many factors, an extract lacking in vitro activity may still possess in vivo activity [29]. Cytotoxicity results obtained in this study will enable the selection of anti-plasmodial extracts and support further investigation for their potential as sources for lead anti-malarial compounds. Most of the tested plants revealed that selectivity indices were high, thus enabling achievement of therapeutic doses at safe concentrations.

Conclusions

We have demonstrated the anti-plasmodial and anti-malarial effects of seven selected medicinal plants commonly used by the Meru community for the treatment of malaria. The aqueous extracts are generally less toxic than methanol ones and may explain the use of the former in traditional systems of health care delivery. A large number of the tested samples had appreciable anti-plasmodial activity. This demonstrates that the ethno-pharmacological approach used in this study may be useful in the search for new anti-malarial drugs from natural sources. Bioassay-guided fractionation of the anti-plasmodial extracts is underway in order to isolate and identify the active compounds. These will be used as markers for standardization of herbal remedies or may lead to anti-malarial compounds for drug development or templates for synthetic drugs. Cytotoxicity evaluation of a few of the selected plants used in the study revealed low selectivity indices especially for methanol extracts. This is an indication of toxic principles contained in them. We recommend that thorough toxicological studies be done on these plants before they are prescribed for human use.

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