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In vivo antimalarial activity of aqueous extracts from Kenyan medicinal plants and their interactions with chloroquine

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Fifteen hot water extracts prepared from 8 plants representing 7 families used traditionally in malaria treatment in Kenya were screened for their *in vivo* antimalarial activity against chloroquine (CQ)-tolerant rodent malaria parasite *Plasmodium berghei* (strain NK65), in ICR mice. When used alone, *Ficus sur* leaf extract had 41% suppression of parasitemia relative to untreated controls (P= 0.002). Four plants, *Albizia gummifera, Caesalpinia volkensii, Ekebergia capensis* and *Maytenus acuminata* showed mild parasitaemia suppression ranging from 9-32%. Three plants, *Ajuga remota, Azadirachta indica* and *Clerodendrum myricoides* showed no activity at all. In combination with CQ, both *F. sur* leaf and stem bark extracts gave a 2-week longer survival of mice relative to the CQ-alone treated controls, although the 2 groups had comparable parasitaemia to the CQ controls before treatment. However, the combinations showed no significant reduction in parasite load. *A. gummifera* leaf extract/CQ combination had 2.3-fold decrease in mean parasitaemia (57%) and also prolonged the survival of mice by over 2 weeks, compared to CQ controls. The results of interactions of the 2 plants' extracts with CQ indicate a potentiation effect. We conclude that both *F. sur* and *A. gummifera* warrant further investigations to determine their potential as sources of antimalarial agents.

Key words Antimalarial, Medicinal plants, Chloroquine combination, *Plasmodium berghei* NK65, Synergism.

Introduction

The current malaria control strategy in most countries relies on disease management using chemotherapy, but its impact is seriously hampered by the spread of antimalarial drug resistance.[1] Drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated.[2] The resurgence of malaria is partly attributed to the development of resistance by *Plasmodium falciparum* (the most common human malaria parasite) to the most commonly used antimalarial drugs such as chloroquine (CQ) and sulfadoxine-pyrimethamine.[3] In Kenya, for instance, 61-80% of parasites isolated from malaria cases are now resistant to CQ, and 30% of those treated with CQ are subsequently considered clinical treatment failures.[4] Due to the widespread CQ resistance, the Ministry of Health recommended the use of sulfa-based drugs (SP's) as the first line therapy in 1999. However in 2004, increased clinical resistance to SP's (27-40%) in several parts of the country prompted yet another change of the first line therapy to artemisinin-based combination therapy (ACT's). Such are the scenarios in most malaria endemic countries, especially in sub-Saharan Africa, which bears over 90% of all global malaria burden.[1,5] In an attempt to impede selection of drug resistance, use of monotherapy is being discouraged for most parasitic diseases.[6] In the case of malaria, for instance, not only are novel combinations being tried, but also attempts are being made to enhance the potency and/or even reverse resistance of conventional drugs such as CQ.[7] Although several synthetic molecules have been shown to restore CQ-sensitivity in resistant *P. falciparum* strains,[8] there are almost no documented data on interactions of herbal remedies with conventional antimalarial drugs such as CQ. It is reported that in Madagascar, several medicinal plants are used by the local population in combination with CQ and that the crude or pure alkaloids from these plants significantly enhanced CQ action both *in vitro* and *in vivo*.[9]

In view of the problems associated with antimalarial drug resistance, new drugs or drug combinations are urgently required today for treatment of malaria. Preferably, the new drugs should have novel modes of action or be chemically different from the drugs in current use.[10] Plants have always been considered to be a possible alternative and rich source of new drugs and the WHO has estimated that 80% of the world's population use botanical medicines for their primary health care needs.[11,12] It is estimated that about 20,000 species of higher plants are used medicinally throughout the world.[13] Most of the antimalarial drugs in use today such as quinine and artemisinin were either obtained directly from plants or developed using chemical structures of plant-derived compounds as templates.[3,10] In

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tropical countries, modern medicines may not be available to most of the rural populations, and even if available, the cost of the drugs is usually prohibitive.[14] The limited availability and affordability of pharmaceutical medicines mean that the majority of the populations in developing countries depend on traditional medical remedies.[15] In the present study, hot water extracts of several medicinal plants used as traditional remedies for malaria in Kenya were evaluated *in vivo* using mice for antimalarial activity, alone or in combination with the commercially available antimalarial drug, CQ.

Materials and Methods

Plant materials collection and extraction. Plant specimens were collected in January 2004 from the Mount Kenya Forest in the Nanyuki area in central Kenya and Magadi on the Nguruman Escarpment in Kenya's South Rift Valley. The plants collected were identified by a taxonomic botanist from the East African Herbarium in Nairobi, and voucher specimens were deposited at the Herbarium and in the Department of Pharmacognosy, University of Shizuoka, Japan. Extracts were obtained from 8 plant species representing 7 families and plant parts used for extraction included leaves, seeds, stem bark, root bark or whole plant (Table 1). The samples were catalogued, dried at room temperature under shade, and were ground into powder using an electric mill (Christy & Morris, Chelmsford, England). The powder was packaged into one kg-packs and stored in a cool dry and well-ventilated room until used. Hot water extraction was used in the present study to mimic the procedure used in traditional extractions. For each plant species, aqueous extracts were prepared from the plant parts by boiling 10 g of the powder in 500 ml of distilled water for 1 hour followed by filtration of the extract. The filtrate was then concentrated and freeze-dried to give a dry sample, which was stored at -20 ℃. Before use, all the extracts were reconstituted with distilled water.

The test parasite. To evaluate the antimalarial activity of the plant extracts, an induced CQ-tolerant malaria parasite *Plasmodium berghei* (strain NK65), maintained at the Parasite Bank of the Department of Parasitology, Hamamatsu University School of Medicine was used. The parasite was previously a kind gift from Professor Y. Wataya of Okayama University, Japan. The cryopreserved parasite specimen was thawed out and inoculated intraperitoneally into 7-week old male outbred SPF ICR mice (Japan SLC Inc., Hamamatsu, Japan) weighing about 30 g, which then served as donor mice for experimental infections. Six days after parasite inoculation, blood was obtained from the donor mice and parasitaemia was assessed microscopically by thin blood smears. Erythrocyte density in blood was estimated on a haemocytometer, the total number of parasitisized erythrocytes (PE) determined, and adjusted downwards with phosphate-buffered saline to give the required number of PE per mouse. ICR mice were each inoculated intraperitoneally with 0.2 ml of diluted blood containing approximately 1 x 10^5 PE to initiate a *P. berghei* infection for antimalarial activity testing.[16,17] The parasite-inoculated mice were randomized into groups of 5 mice per cage. The mice were maintained in an animal care facility at a temperature of 22 ℃, relative humidity of 50-70%, and on a commercial diet (LabDiet, PMI Nutrition International, Mo., USA) and water *ad libitum.*

***In vivo* evaluation of plant extracts for antimalarial activity either alone or in combination with chloroquine (CQ).** The plant extracts were tested for antimalarial activity alone using the 4-day suppressive protocol described by Peters *et al.*[18] The initial (day 0) treatment of the experimental groups was done between 2-4 hours post-infection (p.i.) with the parasite, with each mouse receiving the test extract at a dose of 500 mg/kg body weight, twice a day for 4 days by the oral route. The untreated control group received a corresponding volume of distilled water only. On day 4 p.i. (i.e., 24 hours after the last treatment), thin blood smears were prepared, stained with Giemsa and parasitaemia was determined microscopically by counting the number of parasites in 5 fields of approximately 100 erythrocytes per field.

The method of Ishih *et al.,*[19] where the treatment starts at day 4 p.i., was used to determine whether or not any of the plant extracts produced synergistic effects with CQ (a drug with proven antimalarial properties) *in vivo*. It has been suggested that parasite clearance depends on both initial parasite load, starting day of treament as well as chloroquine (CQ) dose.[20] Ishih *et al.*,[19,20] previously had established that a CQ dose of 20 mg/kg body weight for 2 days (the recommended curative dose) administered to mice from day 0 after intraperitoneal infection with 10^5 *P. berghei* parasites entirely clears the parasites. However, treament with a similar CQ dose after delaying drug administration up to day 4 p.i. either does not or partially clears the parasites. The approach is therefore useful in investigating combined effects of CQ with other drugs, in this case the plant extracts, on parasite clearance. An experiment consisting of infected mice randomized into a CQ group, CQ/plant-extract groups and water control group was set up. Before initial treatment (day 4 p.i.), thin blood smears were prepared for all mice. For all groups, except for the water control group, CQ solution at a dose of 20 mg/kg body weight, once a day for 2 days was orally administered. Except for CQ- and water-control groups, CQ dosage was immediately followed by an oral administration of plant's water extract at a dose of 500 mg/kg body weight, twice a day for 4 days.

In both drug-alone and CQ combination experiments, antimalarial activity was assessed by monitoring mouse survival daily and parasitaemia over a period of 30 days. Parasitaemia was monitored by microscopy on Giemsa stained thin blood smears prepared from mouse tail blood after every 2 days in the course of monitoring period. Mice that survived beyond this monitoring period were killed followed by examination of the liver and spleen organs.

Data and statistical analysis. The percentage suppression of parasitaemia for each plant fraction was calculated as: 100 - [(mean parasitaemia treated/mean parasitaemia control) 100].[16] For comparison of mean parasitaemia, F-

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test (two-sample for variances) and Student's *t*-test (2-tailed) were employed (Microsoft® Excel 2004), with P<0.05 being considered significant.[21]

Ethical considerations. The handling and care of animals was done as recommended in the *Guide for the Care and Use of Laboratory Animals* manual of Hamamatsu University School of Medicine.

Results

***In vivo* antimalarial activity of plant extracts alone.** Each plant part yielded sufficient quantities of dry material after extraction and freeze-drying, to allow their testing for antimalarial activity *in vivo*, in mice (Table 1). Based on the results of mouse survival and day 4 parasitaemia, it was observed that some extracts had mild to remarkable activity. Out of 8 plants tested, one plant, *Ficus sur*, showed remarkable antimalarial activity, 4 plants (*Albizia gummifera. Caesalpinia volkensii, Ekebergia capensis, Maytenus acuminata*) were mildly active, and 3 plants (*Ajuga remota, Azadirachta indica, Clerodendrum myricoides*) did not show any activity (Table 2). Although leaf, stem and root bark extracts of *F. sur* were tested, the leaf extracts were the most potent suppressing parasitaemia by 41%, which was statistically significant (P = 0.002). In the case of *E. capensis* both leaf and root bark extracts had moderate antimalarial activity suppressing parasitaemia by 23% and 32%, respectively. The root bark extract of *F. sur* and leaf extracts of *A. gummifera* and *M. acuminata* were mildly potent with parasitaemia suppression of 14-18%.

In terms of survival, all of the mice in the untreated controls died by day 9 p.i. In contrast, the group treated with stem bark extracts of *Ficus sur* had a 50% mouse survival on day 11 p.i. with the last mouse surviving up to day 19 p.i. Also, mice treated with leaf extracts of *E. capensis* survived longer than the controls with 50% survival on day 12 p.i., and the last mouse surviving up to day 22 p.i. Overall, there was no remarkable difference in survival between mice treated with the extracts and the untreated control groups that received distilled water (Table 2).

***In vivo* antimalarial activity of plant extracts in combination with chloroquine (CQ).** In drug combination studies, mice were treated from day 4 p.i., and both survival and parasite load reduction were assessed with respect to CQ

Table 1 Plants parts collected and percentage yield of the freeze-dried aqueous extract per 10g of dry plant material used

Plant Family/Botanical name (Specimen code)	Plant part used	% Yield of plant extract
Caesalpinaceae		
Caesalpinia volkensii Harms		
(Cv-L/04)	Leaves	22
(Cv-SD/04)	Seeds	21
Celastraceae		
Maytenus acuminata (L.f.) Loes.		
(Ma-L/04)	Leaves	21
Labiatae		
Ajuga remota Benth.		
(Aj-WP/04)	Whole plant	36
Meliaceae		
Ekebergia capensis Sparrm.		
(Ec-L/04)	Leaves	16
(Ec-SB/04)	Stem bark	33
(Ec-RB/04)	Root bark	46
Azadirachta indica A. Juss.		
(Ai-L/04)	Leaves	29
Mimosaceae		
Albizia gummifera (JF Gmel.) C.A. Sm.		
(Ag-L/04)	Leaves	8
(Ag-SB/04)	Stem bark	9
Moraceae		
Ficus sur Forssk.		
(Fs-L/04)	Leaves	15
(Fs-RB/04)	Root bark	12
(Fs-SB/04)	Stem bark	8
Verbenaceae		
Clerodendrum myricoides (Hochst.)	Vatke	
(Cm-L/04)	Leaves	14
(Cm-RB/04)	Root bark	59

Table 2 Parasitaemia suppression and mouse survival after treatment with plant extract

Drugs	Parasitaemia suppression (%) on day 4 p.i	Mouse survival (%) on day 9 p.i.
Ajuga remota		
Whole plant	NS	20
Albizia gummifera		
Leaves [a]	18	25
Stem bark	NS	20
Azadirachta indica		
Leaves [a]	NS	25
Caesalpinia volkensii		
Leaves [a]	NS	25
Seeds	9	20
Clerodendrum myricoides		
Leaves	NS	20
Root bark	NS	0
Ekebergia capensis		
Leaves [a]	23	50
Stem bark [a]	NS	25
Root bark [a]	32	25
Ficus sur		
Leaves	41 [b]	20
Stem bark [a]	23	50
Root bark [a]	14	0
Maytenus acuminata		
Leaves	18	20
Water control		0

Infected mice were treated from day 0 post-infection (p.i.) with aqueous extracts of plants at a dose of 500 mg/kg, twice a day for 4 days.
[a] number of mice per group, n=4, other groups had 5 mice per group
[b] P=0.002, which is significant (p<0.05 was considered significant)
NS, no suppression

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Table 3 Parasitaemia suppression and mouse survival after treatment with plant extract in combination with chloroquine (CQ)

Drugs	Parasitaemia suppression (%) on day 11 p.i	Mouse survival (%) on day 14 p.i.
Ajuga remota		
Whole plant	NS	40
Albizia gummifera		
Leaves	57 [b]	40
Stem bark	23	20
Azadirachta indica		
Leaves	NS	20
Caesalpinia volkensii		
Leaves [a]	7	25
Seeds	3	40
Clerodendrum myricoides		
Leaves	33	40
Root bark	23	40
Ekebergia capensis		
Leaves	17	0
Stem bark	40	40
Root bark	10	40
Ficus sur		
Leaves	NS	60
Stem bark	10	40
Root bark	7	20
Maytenus acuminata		
Leaves [a]	23	25
Chloroquine (control)		0

Infected mice were treated with CQ (at a dose of 20 mg/kg, once a day for 2 days) from day 4 post-infection (p.i.) followed by aqueous extracts of plants (at a dose of 500 mg/kg, twice a day for 4 days).
[a]number of mice, n=4, for all other groups, n=5.
[b]P=0.008 which is significant ($p<0.05$ was considered significant)
NS, no suppression

treated controls. Table 3 summarizes the results of percentage parasitaemia suppression on day 11 p.i. and % mouse survival on day 14 p.i. All mice in the CQ-alone treated control group died between day 13 and 14 p.i. Out of the 8 plants whose extracts were assayed in combination with CQ, 3 plants (*A. gummifera, F. sur, E. capensis*) had remarkable activity with respect to parasitaemia suppression and/or long survival of mice, relative to CQ-alone treated group. Three plants (*C. volkensii, C. myricoides, M. acuminata*) had low to mild activity with respect to parasitaemia suppression that ranged from 3-33%. However, *C. volkensii* (seeds), *C. myricoides* (root bark and leaves) had significant number of mice (40%) surviving far much longer than the CQ-treated control group. Two plants, *A. remota* and *A. indica* exhibited no parasitaemia suppression relative to CQ control group. For all groups, no parasites were observable in thin smears by microscopy on day 8 p.i. However, by day 11 p.i. (i.e 4 days after the last treatment), recrudescent parasites were observable in thin smears. Although the mice in CQ-treated control group died between day 13 and 14 p.i., the group that received CQ/*F. sur* leaf extract, had 60% survival up to day 28 p.i., with the last mouse surviving up to day 30 p.i. The group treated with CQ/*F. sur* stem bark extract had 40% survival up to day 21 p.i., with the last mouse surviving up to day 27 p.i. Similarly, the group treated with CQ/*A. gummifera* leaf extract had 40% survival up to day 28 p.i., with the last mouse of the group surviving up to day 30 p.i. The survival periods of mice treated with extracts of these two plants in combination with CQ were about twice that of CQ control group. In addition to prolonged mouse survival, CQ/*A. gummifera* leaf extract combination also gave parasitaemia suppression of 57% on day 11 p.i relative to CQ-treated controls (P=0.008). This is despite the fact that the 2 groups had comparable parasitaemia (day 4) before initial treatment (P=0.67). Conversely, although CQ/*F. sur* leaf extract group had the highest survival rate, no significant reduction of parasite load was observed on day 11 p.i. between this group and CQ controls (P=0.36), although the 2 groups had comparable parasitaemia before the initial treatment (P=0.35).

Ekebergia capensis stem bark extract in combination with CQ gave 40% parasitaemia suppression relative to CQ-alone treated mice. Although this suppression is not statistically significant (P=0.20), it is remarkable that both the plant's stem and root bark extracts in combination with CQ had 40% surviving mice on day 14 p.i., which died between day 15 and 29 p.i. Mice in the other groups that survived beyond day 14 p.i. mostly died by day 18 p.i.

Discussion and Conclusions

Ficus sur leaf extract, when used alone, showed remarkable antiplasmodial activity with regard to parasite reduction. In combination with CQ, the plant's leaf extracts showed no suppression, while the stem bark extracts gave a mild suppression. However, both extracts in combination with CQ gave a longer survival of mice than the CQ controls. *Albizia gummifera* leaf extract when used alone had no significant suppression of parasitaemia. However, in combination with CQ, there was a remarkable suppression of parasitaemia. These results, where the extracts showed either no suppression of parasitaemia and/or prolonged mouse survival when used alone, but showed the same when used in combination with CQ suggest that although direct parasiticidal activity may be involved, other indirect pharmacological effects of the plant extracts, such as stimulation of the host's immune system may not be ruled out. On the other hand, the significant activity of *F. sur* leaf extract is apparently lost when used in combination with CQ, which strongly suggest that there may be cases of antagonistic interactions between CQ and the plant extracts, and therefore use of conventional drugs with natural products before the safety and efficacy of such combinations are verified should be discouraged. In combination of 2 or more drugs, synergistic interactions are anticipated. However there are cases where interactions of drugs in combination may be antagonistic and/or toxic. For instance, antifolates have been reported to possess both *in vitro* and *in vivo* antagonistic interactions with artemisinin.[22,23] All the plant extracts tested were generally well tolerated by mice even at doses of as high as 1000 mg/kg body weight per day for 4 days. None of the treated mice died within 48 hr of extract

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administration, ruling out acute toxicity, and since examination of livers and spleens of treated mice did not reveal any obvious organ damage, the mortality of mice observed from day 7 p.i. was therefore attributed to increased parasitaemia, and not to toxicity of the extracts. The fact that the plant extracts, mainly those of *F. sur* and *A. gummifera* showed antiplasmodial activity, prolonged mouse survival or had a potentiation antiplasmodial effect with CQ and were well tolerated by mice may partly validate the use of the plants in ethno-medicine.

In a previous study, either aqueous and/or organic extracts of *A. remota, F. sur, E. capensis* or *C. myricoides,* which had been collected from the western part of Kenya, were shown to have mild to remarkable antiplasmodial activity *in vitro* against CQ-resistant and CQ-sensitive *P.falciparum* isolates, either alone and/or in combination with CQ.[24,25] Interestingly, in the present study, extracts of *A. remota* and *C. myricoides* showed no *in vivo* activities. Malaria may manifest with symptoms such as fever, joint pains and suppression of the immune system. Therefore, further investigation on the plants to determine their potential value in malaria therapy is recommended since plants' bioactive compounds may not necessarily possess direct parasiticidal effect but may have other pharmacological properties such as antipyretic, analgesic or immunostimulatory.[26,27] It is also known that the presence or quantities of bioactive compounds in plants may be influenced by several factors including season, weather conditions, environment, plant-part used, intra-species variations and plant age, among other factors.[26] This underlines the necessity to domesticate or cultivate medicinal plants as has been done with *Artemisia annua* and *Cinchona* species, plants that in the past have provided antimalarial compounds which have been used as templates in development of novel antimalarials.[28] Also, the nature of the solvent used and traditional extraction methods such as direct boiling of plant material are significant factors worthy of re-evaluation. Although in traditional usage water is the solvent of choice, some bioactive compounds may be non-polar in nature. In this regard, we are currently evaluating the organic extracts of the plants of this study for antimalarial activity, which will ultimately lead to isolation and characterization of the bioactive compounds. It is imperative for researchers to sufficiently explore medicinal plants with the object of discovering alternative and affordable antimalarial drugs, in the fight against drug resistant *P. falciparum* malaria.

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Japanese abstract

ケニアにおいて伝統的にマラリア治療に用いられている7科に属する8種の植物から15種類の水煮沸抽出液を調製し，これらの抗マラリア活性についてクロロキン低感受性のネズミマラリア原虫 *Plasmodium berghei* NK65を感染させたICRマウスを用いて検討した。抽出液単独投与では，*Ficus sur* の葉抽出液が，未治療感染対照群の血虫率に対し41%の有意な抑制効果を示した。4種の植物，*Albizia gummifera, Caesalpinia volkensii, Ekebergia capensis* そして *Maytenus acuminata* 投与では，9〜32%の抑制が認められた。3種の植物 *Ajuga remota, Azadirachta indica* そして *Clerodendrum myricoides* は活性を示さなかった。一方，クロロキンと抽出液の併用では，*Ficus sur* の葉および樹皮の抽出液投与では，原虫増殖の有意な抑制は認められなかったが，クロロキン投与対照群の生存期間に対して2週間の延長が認められた。また *Albizia gummifera* の葉抽出液とクロロキンとの併用で有意な血虫率の減少が認められ，さらに2週間以上の生存期間の延長が認められた。これら2種の植物から調製した抽出液とクロロキンとの相互作用は相乗作用を示唆している。抗マラリア薬開発の材料としての可能性を探る意味で，ケニア原産植物の *Ficus sur* および *Albizia gummifera* の抗マラリア活性成分の更なる研究が望まれる。

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