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In vitro antiplasmodial activity of some plants used in Kisii, Kenya against malaria and their chloroquine potentiation effects

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Abstract

Fifty-five organic and aqueous extracts of 11 plants used in malaria therapy in Kisii District, Kenya were tested in vitro against chloroquine (CQ)-sensitive and resistant strains of *Plasmodium falciparum*. Of the plants tested, 73% were active ($IC_{50} < 100$ μg/ml). Three plants, *Vernonia lasiopus*, *Rhamnus prinoides* and *Ficus sur* afforded extracts with IC_{50} values ranging less than 30 μg/ml against both CQ-sensitive and resistant strains. Combination of some extracts with CQ against the multi-drug resistant *P. falciparum* isolate V1/S revealed some synergistic effect. The plant extracts with low IC_{50} values may be used as sources for novel antimalarial compounds to be used alone or in combination with CQ.

Keywords: Plant extracts; Antiplasmodial; Medicinal plants; *Plasmodium falciparum* synergistic effect

1. Introduction

Plasmodium falciparum, the most widespread etiological agent for human malaria is becoming increasingly resistant to standard antimalarial drugs which necessitates a continuous effort to search for new antimalarial drugs. Plants have invariably been a rich source for new drugs and some antimalarial drugs in use today (quinine and artemisinine) were either obtained from plants or developed using their chemical structures as templates (Gessler et al., 1994). Scientific evaluation of medicinal plants used in the preparation of traditional medicine has in the past provided modern medicine with effective drugs for the treatment of parasitic diseases (Iwu et al., 1994).

Over the past years, the scientific progress in malaria chemotherapy has been more on the effects of already existing drugs than the development of new ones. The search for new antimalarial drugs has regained importance due to resurgence of drug resistant parasites in many countries. Malaria chemotherapy and prophylaxis are now targeting drug combinations with the hope of achieving drug potentiation to circumvent or delay resistance (Zucker and Campbell, 1993; WHO, 2000). In South East Asia, the use of firstline drugs with artemisinine derivates such as artesunate have shown to increase efficacy, protect drugs against resistance development, reduce transmission of malaria and increase the lifespan of antimalarial compounds (TDR, 2000). In Kenya, chloroquine (CQ) has been discontinued as the firstline treatment for malaria due to overwhelming presence of resistant *P. falciparum* strains. Reports of resistance to sulfadoxine-pyrimethamine, which is cur-

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rently used as the firstline drug, are on the increase (WHO, 1998, 2000; USN, 2001).

2. Materials and methods

2.1. Plant materials

The plant samples were collected between March and September 1999 from Kisii District, Nyanza Province, Kenya based on ethnomedical use through interviews with local communities (Table 1). These were identified and voucher specimens deposited at the Herbarium, Botany Department, University of Nairobi, Nairobi. The plant parts were air-dried under shade and ground using a laboratory mill (root bark and stem bark) and a kitchen blender (leaves).

2.2. Extraction

For each plant part, 25 g of the chaff was boiled for 5 min in 250 ml of water followed by thorough mixing (shaker) and soaking for 12 h. The extract was filtered and freeze-dried to give 1–3 g of dry solid. Sequential organic extraction was also done by soaking the plant chaff for 48 h, using solvents of increasing polarity (hexane, chloroform, ethyl acetate, methanol). The solvent was removed under reduced pressure at 40 °C and the residue was dried under vacuum over anhydrous silica gel to give a dry solid or paste.

Table 1
Plant parts collected based on ethnomedical data

Family/botanical name	Vernacular	Plant part
Acanthaceae		
Justicia betonica L. (MP-SM/1/99)	Mokera ogesimba	Aerial parts
Compositae		
Aspilia pluriseta Schweinf. (MP-SM/237/99)	Rirangera	Leaves
Microglossa pyrifolia (Lam.) O.Kuntze (MP-SM/24/99)	Mote O' Kebaki	Leaves
Vernonia auriculifera (Welw.) Hiern (MP-SM/28/99)	Omosabakwa	Leaves
V. lasiopus O.Hoffm. (MP-SM/30/99)	Omosoricho	Leaves
Euphorbiaceace		
Euphorbia inaequilatera Sond. (MP-SM/37/99)	Ogota Kwembeba	Whole plant
Moraceae		
Ficus sur Forssk. (MP-SM/67/99)	Omora	Stem bark
Rhamnaceae		
Rhamnus prinoides L'Hérit. (MP-SM/72/99)	Omonguroro	Root bark
R. staddo A.Rich. (MP-SM/75/99)	Omontontono	Root bark
Rubiaceae		
Spermacoce princeae (K.Schum.) Verdc. (MP-SM/78/99)	Omonhabiebo	Whole plant
Urticaceae		
Urtica massaica Mildbr. (MP-SM/82/99)	Rise	Aerial parts

2.3. Preparation of drugs

Stock solutions of crude extracts (250 μg/ml) were made with sterile water (deionized and autoclaved) and consecutively filtered first through 0.45 and 0.22 μm microfilters in a laminar flow hood. The water insoluble extracts were first dissolved in dimethylsulfoxide (solvent concentration $<0.02\%$) (Elueze et al., 1996). A stock solution of CQ phosphate (1 μg/ml) was similarly prepared in sterile water. All the drug solutions were stored at −20 °C until used.

2.4. Cultures of Plasmodium falciparum

Laboratory-adapted *P. falciparum* cultures of K39 (CQ-sensitive isolate, originally obtained from a patient in Kisumu, Kenya) and ENT 30 (CQ-resistant isolate, originally obtained from a patient from Entosopia, Kenya), the international reference isolates NF 54 (CQ-sensitive) and VI/S (CQ-resistant) were used in this study. The strains have been cultured and maintained at the Malaria Laboratories of Kenya Medical Research Institute (KEMRI), Nairobi. The culture medium was a variation of that described by Trager and Jensen (1976) and consisted of RPMI 1640 supplemented with 10% human serum, 25 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid and 25 mM $NaHCO_3$. Human type O+ erythrocytes (<28 days old) served as host cells and the cultures were incubated at 37 °C in an atmosphere of 3% CO_2, 5% O_2 and 92% N_2.

2.5. Bioassays

The in vitro semi-automated microdilution assay technique that measured the ability of the extracts to inhibit the incorporation of [G-^{3}H]hypoxanthine into the malaria parasite was used (Desjardins et al., 1979; Omulokoli et al., 1997). For the test, 25 μl aliquots of culture medium were added to all the wells of a 96 well flat-bottom microculture plate (Costar Glass Works, Cambridge, UK). Aliquots (25 μl) of the test solutions were added, in duplicate, to the first wells, and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK) was used to make serial 2-fold dilutions of each sample over a 64-fold concentration range. Aliquots (200 μl) of a 1.5% (vol.–vol.) suspension of parasitized erythrocytes in culture medium (0.4% parasitemia; growth rate >3-fold per 48 h) were added to all

test wells. Parasitized and non-parasitized erythrocytes were incorporated into all tests. The plates were incubated at 37 °C in a gas mixture 3% CO_2, 5% O_2 and 92% N_2. After 48 h each well was pulsed with 25 μl of culture medium containing 0.5 μCi of [G-^{3}H]hypoxanthine and the plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filters, washed thoroughly with distilled water, dried and the radioactivity in counts per minute (cpm) measured by liquid scintillation.

Computation of the concentration of drug causing 50% inhibition of [G-^{3}H]hypoxanthine uptake (IC_{50}) was carried out by interpolation after logarithmic transformation of both concentration and cpm values using the formula, $IC_{50} = \text{antilog}\ (\log X_1 + [(\log Y_{50} - \log Y_1)(\log X_2 - \log X_1)]/(\log Y_2 - \log Y_1)])$, 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 (Sixsmith et al., 1984).

2.6. Drug interaction experiments

The method described by Canfield et al. (1995) was used. The solutions of initial concentrations 20–50 times the estimated IC_{50} values were combined in various ratios of antimalarial drugs. Single and combined drug solutions were dispensed into 96 flat-bottomed well microtitre plates to give duplicate rows of CQ alone, the test drug (plant extract), and the nine combinations. The ratios were spread into nine combinations ranging from 90:10 to 10:90 (CQ:extract) (Fivelman et al., 1999). Incubation and subsequent procedures were followed as described earlier. The results were expressed as the sum of the fractional inhibitory concentration (sum FIC) (Berenbaum, 1978).

3. Results and discussion

In the preliminary studies, all the 55 extracts from the 11 plants were screened against K39. At least one extract from 73% of all the plants screened against K39, a CQ-sensitive *P. falciparum* isolate, showed activity of IC_{50} values below 100 μg/ml (Table 2). The extracts that were with $IC_{50} < 20$ μg/ml were further assayed against NF 54, ENT 30 and V1/S (Table 3).

For K39, the two plants from Rhamnaceae (*R. prinoides* and *R. staddo*) showed mild antiplasmodial activity with methanol extracts being the best (IC_{50} = 15.05 and 25.64 μg/ml, respectively). Of the 11 plant species investigated members of the family Compositae exhibited the best antiplasmodial activity with *V. lasiopus* giving the lowest IC_{50} values. Both *V. auriculifera* and *M. pyrifolia* yielded extracts within the mild activity range (20–100 μg/ml) while the hexane and methanol extracts of *A. pluriseta* showed mild activity with IC_{50} values of 62.70 and 40.81 μg/ml, respectively. Of the five extracts of *J. betonica*, only the methanol extract had mild activity ($IC_{50} = 69.55$ μg/ml). All the extracts of *E. inaequilatera*, *S. princeae* and *U. massaica* did not show any in vitro antimalarial activity.

For both K39 and NF 54, *V. lasiopus* seem to give comparable IC_{50} values for chloroform, ethyl acetate and methanol extracts (1.21, 1.01 and 3.15 μg/ml for K39, and 1.67, 1.62 and 3.52 μg/ml for NF 54, respectively). There are cases in which both the CQ-sensitive and resistant isolates seem to be equally susceptible to the same extracts. For instance, *V. lasiopus* ethyl acetate extract had comparable IC_{50} values for all the isolates (1.01, 1.62, 1.37, 1.59 μg/ml against K39, NF 54, ENT 30 and V1/S, respectively). The hexane, chloroform and methanol extracts of the leaves of *V. lasiopus* did not show any appreciable bioactivity by the brine shrimp lethality test (Meyer et al., 1982).

The extracts of *V. lasiopus*, which exhibited IC_{50} values ≤ 5 μg/ml singly were combined with CQ and tested against the multi-drug resistant *P. falciparum* isolate, V1/S (Table 4). Except for a few drug combinations, CQ and *V. lasiopus* extracts exhibited good synergy.

The best extracts against K39 were those of *V. lasiopus* which gave IC_{50} values of 1.21, 1.01 and 3.15 μg/ml from chloroform, ethyl acetate and methanol extracts, respectively. These values are nearly double the IC_{50} values of the crude extracts of *Cinchona* species ($IC_{50} = 0.5$ μg/ml) against *P. falciparum* (Weenan et al., 1990). Further, screening the extracts which showed low IC_{50} values with K39, NF 54, ENT 30 and V1/S confirm that the extracts had good antiplasmodial activity. *V. lasiopus* extracts exhibited very low IC_{50} values for all the four isolates. The chemistry and biological activity of other *Vernonia* species such as *V. brachycalyx* and *V. amygdalina* have been reported (Oketch-Rabah, 1996) In these studies, in vitro antiplasmodial activity of *V. brachycalyx* chloroform: ethyl acetate (1:1) crude extract (leaves) had the best activity against K39 (IC_{50} = 6.62 μg/ml) and V1/S ($IC_{50} = 8.43$ μg/ml) while the methanol extract had IC_{50} values of 29.62 and 16.64 μg/ml against K39 and V1/S, respectively. The aqueous extract showed the lowest activity with IC_{50} values of 31.22 and 30.22 μg/ml against K39 and V1/S, respectively (Oketch-Rabah, 1996). These findings are consistent with the results of the present study in which the best activity were those of the ethyl acetate and chloroform extracts against all the four *P. falciparum* isolates used. The activity of the chloroform, ethyl acetate and methanol extracts of *V. lasiopus* ranged from 1.01 to 4.13 μg/ml for both the CQ-sensitive and resistant isolates. Although several compounds were isolated

test wells. Parasitized and non-parasitized erythrocytes were incorporated into all tests. The plates were incubated at 37 °C in a gas mixture 3% CO_2, 5% O_2 and 92% N_2. After 48 h each well was pulsed with 25 μl of culture medium containing 0.5 μCi of [G-^{3}H]hypoxanthine and the plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filters, washed thoroughly with distilled water, dried and the radioactivity in counts per minute (cpm) measured by liquid scintillation.

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Table 2
The mean IC_{50} values ($x \pm$S.D.) for plant extracts screened against *P. falciparum* isolate K39

Plant species	$IC_{50} \pm$S.D. (μg/ml)				
	Hexane	Chloroform	Ethyl acetate	Methanol	Water
Aspilia pluriseta	62.7±8.9	>100	>100	40.8±5.7	100
Euphorbia inaequilatera	>100	>100	>100	>100	>100
Ficus sur	19.2±1.3	9.0±3.2	>100	>100	>100
Justicia betonica	>100	>100	>100	69.6±1.9	>100
Microglossa pyrifolia pyrifolia	72.5±2.5	58.4±1.9	79.5±2.7	68.4±3.5	>100
Rhamnus prinoides	>100	96.8±1.0	50.7±3.0	15.1±2.3	82.3±2.44
R. staddo	>100	>100	35.2±3.7	25.6±2.4	>100
Spermacoce princeae	>100	>100	>100	>100	>100
Urtica massaica	>100	>100	>100	>100	>100
Vernonia auriculifera	>100	37.7±1.3	40.3±2.0	55.2±2.3	>100
V. lasiopus	>100	1.2±0.1	1.0±0.1	3.2±0.3	>100

CQ IC_{50} value = 0.021 μg/ml and was included as positive control.

Table 3
The mean IC_{50} values ($x \pm$S.D.) for selected plant extracts against *P. falciparum* isolates K39, NF 54, ENT 30 and V1/S

Extracts	$IC_{50} \pm$S.D. (μg/ml)			
	CQ-sensitive isolates		CQ-resistant isolates	
	K39	NF 54	ENT 30	V1/S
Ficus sur				
Hexane	19.2±1.3	>62.5	27.4±6.9	>100
R. prinoides				
Methanol	15.1±0.3	>62.5	23.2±1.8	29.9±1.8
V. lasiopus				
Chloroform	1.2±0.1	1.7±0.3	3.6±1.0	3.4±0.3
Ethyl acetate	1.0±0.1	1.6±0.4	1.4±0.1	1.6±0.9
CQ (μg/ml)[a]	0.021	0.016	0.066	0.073

[a] CQ is included as a positive control.

Table 4
Interactions of CQ with *V. lasiopus* extracts against multi-drug resistant *P. falciparum* isolate V1/S

Combination ratios	Sum FIC		
	$CHCl_3$	EtOAc	MeOH
90:10	0.16	0.55	0.24
80:20	0.20	0.32	0.31
70:30	0.14	0.31	0.17
60:40	0.15	0.26	0.17
50:50	0.32	0.29	0.11
40:60	0.16	0.29	0.14
30:70	0.15	0.31	0.73
20:80	0.13	1.00	1.17
10:90	0.97	1.24	1.84

FIC values <1:synergism, FIC values >1:antagonism, FIC values = 1:addition.

from *V. brachycalyx*, 16,17-dihydrobrachycalyxolid was shown to be the major antiplasmodial principle with IC_{50} values of 4.2 and 13.7 μg/ml against K39 and 3d 7 (both CQ-sensitive), 3.0 and 16.1 μg/ml against V1/S and Dd 2 (both CQ-resistant), respectively (Oketch-Rabah et al., 1998). The crude extracts of *V. lasiopus* exhibited good antiplasmodial activity comparable to those of *Cinchona* (0.5 μg/ml) and even better than the active principle of *V. brachycalyx*. It would be interesting to investigate this plant for novel antiplasmodial compounds.

Rhamnus prinoides showed moderate activity (IC_{50} = 15.05–96.80 μg/ml). This plant warrants detailed study, especially the methanol extract, which had IC_{50} value of 15.05 μg/ml against K39. *Ficus sur* was the only plant that had an active hexane extract (IC_{50} = 19.15 μg/ml). The chloroform extract (IC_{50} = 8.99 μg/ml) was also amongst the highest active against K39 and therefore the plant warrants further detailed biochemical studies.

Of the 11 plant species screened against K39, only 3 plant species showed no antiplasmodial activity (IC_{50} > 100 μg/ml) for all of the extracts (*S. princeae*, *E. inaequilatera* and *U. massaica*). The lack of antiplasmodial activity in these plants may not necessarily imply the same in vivo since compounds may either act as pro-drugs (which must undergo metabolic changes to achieve the required activity), febrifuges (fever is one of the symptoms associated with uncomplicated severe *P. falciparum* malaria) or immuno-modulators. Besides the presence of bioactive compounds depends on many factors such as the season, age, intra-species variation, part collected, soil and climate. Therefore, lack of in vitro activity in this case does not disqualify the use of these plants as traditional antimalarials. While plant extracts may not display in vitro activity they may display in vivo activity (Gessler et al., 1995) or vice versa. It is, therefore, necessary to undertake in vivo

investigation of these plants before any conclusion on their efficacy as antimalarials could be drawn.

V. lasiopus extracts potentiated CQ at all combinations except for a few which showed antagonistic or additive effects. Several synthetic molecules have been shown to restore CQ-sensitivity in resistant *P. falciparum* strains (Oduola et al., 1998). However, very little work has been done on the reversal of CQ-resistance using herbal remedies. Rasoanaivo et al. (1992) investigated several medicinal plants used by local populations in Madagascar in association with CQ. They have shown that the crude or pure alkaloids significantly enhance CQ action both in vitro and in vivo.

4. Conclusion

The fact that 73% of the plants screened in vitro had some level of antiplasmodial activity would justify their ethnopharmacological uses as traditional antimalarials. After detailed in vivo antimalarial evaluation and thorough toxicological studies, some of these plants may be recommended as antimalarials in known dosages especially in rural communities where the conventional drugs are unaffordable or unavailable and the health facilities inaccessible. Similarly, some of these plants like *V. lasiopus* may be recommended for CQ potentiation after detailed scientific evaluation in vivo. Biochemical investigations on some of these plants may provide useful templates for the development of novel antimalarial drugs which are effective against CQ-resistant *P. falciparum* strains.

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