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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

In vitro and *in vivo* antiplasmodial activity of extracts of selected Kenyan medicinal plants

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Malaria is one of the most important parasitic diseases in the world. It remains a major public health problem in Africa and is responsible for the annual death of over one million children below the age of five years. The *Plasmodium falciparum* is the most widespread etiological agent for human malaria and has become increasingly resistant to standard antimalarial drugs. Therefore, this necessitates a continuous effort to search for new drugs, particularly with novel modes of action. The medicinal plants have invariably been a rich source for new drugs and some antimalarial drugs in use today (quinine and artemisinin) were either obtained from plants or developed using their chemical structures as templates. The aim of the study was to screen seven selected medicinal plants from Kenya for antimalarial activity. 21 extracts from seven plants, were selected for *in vitro* antimalarial screening out of which 10 extracts with good activity *in vitro* were tested further in a mouse model. *In vitro* antiplasmodial testing was done by measuring the ability of the test sample to inhibit the incorporation of radio-labelled hypoxanthine into the malaria parasite. *In vivo* bioassay was done in mice using Peter's 4-days suppressive test. From the results achieved, 10 out of the 21 extracts tested, exhibited antiplasmodial activity, with IC_{50} values ranging from 6.93 to 88.4 µg/ml. When tested *in vivo*, one of the extracts had high activity with chemo suppression of 82.17%, while the rest ranged between 39.93 and 61.86%. The activities observed especially with the methanol extracts indicate that these plants deserve to be investigated further as potential antimalarial agents.

Key words: Malaria, antimalarial drugs, medicinal plants, *Plasmodium falciparum*.

INTRODUCTION

Malaria is one of the major threats concerning world public health. There are around 250 million clinical cases every year and almost one million deaths, mostly children, which are attributable to this disease (WHO, 2008). The main reasons that explain this worsening situation are therapy problems: resistance to the current

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antimalarial drugs, unavailability and unaffordability of antimalarial drugs and lack of new therapeutic targets (Muregi et al., 2003). Plants have invariably been a rich source for new drugs and some antimalarial drugs in use today (quinine and artemisinin) were either obtained from plants or developed using their chemical structures as templates (Gessler et al., 1994). It is already estimated that 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001). Plants commonly used in traditional medicine are assumed to be safe due to their long usage in the treatment of diseases according to knowledge accumulated over centuries. Therefore, the search for new drugs through the evaluation and validation of traditional medicines offers a good opportunity for the discovery and development of better medicines. Today, many pharmacological classes of drugs contain a natural product prototype. Morphine one of the best painkillers was isolated from *Papaver somniferum*, aspirin an analgesic and anticoagulant from the willow bark, warfarin an anticoagulant from *Melilotus officinalis*, Metformin the current gold standard for management of type 2 diabetes from *Galega officinalis*, quinine from *Cinchona ledgeria* and artemisinin currently the most potent antimalarial drug from *Artemisia annua* (Gilani and Rahman, 2005; Bailey et al., 2007; Gathirwa et al., 2011). Similarly, the *in vivo* antimalarial properties of several plant extracts have been studied in mice (Andrade-Neto et al., 2003). Following this trend, this study presents the results obtained from the evaluation of the *in vitro* and *in vivo* antiplasmodial activity of seven medicinal plants, namely, *Clerodendrum myricoides, Clutia abyssinica, Asparagus racemosus, Asparagus remota, Carpobrotus edulis*, *Satyrium princeae* and *Leucas calostachys*. These medicinal plants are commonly used in Nandi community in Kenya. Cytotoxicity on human fibroblasts was also evaluated in order to determine the selectivity of antimalarial action.

MATERIALS AND METHODS

Plant collection and preparation

The seven medicinal plants were selected on the basis of their traditional reputation to treat fevers or malaria locally. The plants were collected in Aldai division, Nandi County in March 2006. Permission for a sustainable plant harvesting was obtained from the Kenya Wildlife Services in the forest reserves, and from the local communities outside the forest area. The plant specimens were identified and authenticated and voucher specimens deposited at the Botanical Garden Herbarium of Maseno and East African Herbarium, National Museum of Kenya. *C. myricoides* (PJ/AL/03/06/01), *C. abyssinica* (PJ/AL/03/06/02), *A. racemosus* (PJ/AL/03/06/03), *A. remota* (PJ/AL/03/06/04), *C. edulis* (PJ/AL/03/06/05), *S. princeae* (PJ/AL/03/06/06), and *L. calostachys* (PJ/AL/03/06/07).

The plant parts were air-dried at room temperature under shade for 14 days and pulverized using a laboratory mill (Christy and Norris Ltd., Chelmsford, England) at the Center for Traditional Medicine and Drug Research, Kenya Medical Research Institute. The resulting powders were packed in air tight polythene bags, labeled and stored in the dark until used.

Plant extractions

The plant material (50 g) was extracted at room temperature with chloromethane and methanol for 24 h each. The resulting extracts were filtered and concentrated under reduced pressure *in vacuo* to yield chloromethane extract and methanol extract. Another 50 g of the same sample was extracted once with 500 ml of distilled water in a water bath at 60°C for 1 h, filtered and lyophilized in a freeze dryer. The dry extracts for all the samples similarly treated were weighed into airtight containers and stored at 4°C until used.

In vitro antiplasmodial testing

The Sierra Leonean CQ sensitive (D6) and Indochinese CQ-resistant (W2) *Plasmodium falciparum* strains were used in this study. The parasite cultures were donated by the Malaria Research and Reference Reagent Resource Center (MR4), Manassas, Virginia, USA. Parasite cultivation was carried out using previously described procedures (Trager and Jensen, 1976; Schlichtherle et al., 2000). Cultures were maintained in RPMI 1640 culture medium (10.4 g/L) powdered medium without PABA and lactic acid (LA) dissolved in 960 ml of double distilled autoclaved water (DDAW), supplemented with 10% human serum, 25 mM (5.94 g/L) HEPES and 25 mM $NaHCO_3$. Human O+ red blood cells served as the parasites host cells. Test samples were prepared by dissolving in 100% dimethyl sulfoxide (DMSO) and diluting in RPMI to lower the concentration of DMSO to ≤1%. Stock solution (1 µg/ml) of chloroquine was also prepared for use as the reference drug. Semi-automated micro dilution assay technique that measures the ability of the extracts to inhibit the incorporation of [G-3H] hypoxanthine (Amersham International, Burkinghamshire, UK) into the malaria parasite was used in testing antiplasmodial activity (Desjardins et al., 1979). Aliquots (50 µl) of the test solutions were added in the first wells of 96 well flat bottomed micro culture plates (Costar Glass Works, Cambridge, UK) in duplicate and serial diluted down the plate using a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, UK). The last row of wells did not contain the test samples and served as the controls. Two hundred microliters of malaria parasite culture was added into each well. The set plates were incubated at 37°C in a gas mixture 3% CO_2, 5% O_2 and 92% N_2 for 48 h, after which each well was pulsed with 25 µl of culture medium containing 0.5 µCi of [G-3H] hypoxanthine. The plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filter mats, washed thoroughly with distilled water, dried and the radioactivity in counts per minute (cpm) measured using a beta-counter (Wallac Micro Beta Trilux). The cpms obtained were then used to compute the IC_{50} values as described by Sixsmith et al. (1984).

In vivo antimalarial testing

Male albino mice (6 to 8 weeks old, weighing 20±2 g) were used in the experiment. The mice were housed in standard macrolon type II cages in air-conditioned rooms at 22°C, 50 to 70% relative humidity, fed with the standard diet and received water *ad libitum*. *Plasmodium berghei* strain ANKA was maintained by serial passage of infected blood through interperitonial injection (ip). The test protocol was based on the 4-day suppressive test (Peters et al., 1975). Briefly, *P. berghei* infected blood was obtained by heart puncture, mixed with 1% (w/v) heparin in phosphate buffered saline (PBS) (1:1) and the test animals infected by interperitoneal (ip) injection with 0.2 ml (1×10^7 parasitized erythrocytes). Infected mice

Table 1. The mean IC_{50} values (X ± SD) for plants extracts against D6 µg/ml.

Plant species	IC_{50} (µg/ml)		
	Chloroform	Methanol	Water
C. myricoides			
Leaves	>100	19.96	>100
Rootbark	>100	10.2	>100
L. calostachys leaves	40.2	88.4	>100
A. racemosus leaves	>100	31.35	>125
C. abbysinica			
Leaves	>100	26.72	>100
Roots	>100	6.93	66.52
A. remota (Leaves)	>100	22.1	>100
C. edulis (Roots)	>100	>100	>100
S. princeae	>100	>100	>100

Chloroquine IC_{50} value = 0.020 µg/ml (positive control).

were randomly selected into groups of five for one test sample and the experimental groups treated with a single dose of 800 mg/kg of the test sample in 0.2 ml by oral adminstration from day 0 (immediately after infection) to 3 (Gessler et al., 1995). The mice in the positive control group were given chloroquine diphoshate (dissolved in 10% tween 80) at 5 mg/kg/day orally. Those in the negative control group received the placebo (10% tween 80) at 0.2 ml/kg/day. Parasitaemia was determined on day 4 (24 h after the last treatment) by microscopic examination by counting parasites in 4 fields of ≈100 erythrocytes per view of thin blood film sampled from the tail of the experimental mouse and stained in 10% giemsa solution. The difference between the mean number of parasites per view in the negative control group (100%) and those of the experimental groups was calculated and expressed as percent parasitaemia suppression (chemo suppression) according to the formula: $PS = [(A–B)/A]×100$ (Tona et al., 2001), where A is the mean parasitaemia in the negative control on day 4, and B the corresponding parasitaemia in the test group. All *in vivo* experiments were repeated three times. In cases where the standard deviation (SD) was large, the experiment was repeated yet again and in some instances, outliers were not considered while computing the SD. Percentage parasitaemia was described as number of parasitized erythrocytes per 100 erythrocytes while percentage chemo suppression was taken as inhibition of parasite growth/multiplication relative to control expressed in percentage. Parasites in the negative control group were assumed to have experienced 0% chemo suppression. Chemo suppression is thus the potency of the drug to inhibit parasite growth/multiplication.

RESULTS

In vitro antiplasmodial assays

The *in vitro* activities of *L. calostachys, A. remota* and *A. racemosus* were within the mild activity range (20 to 100 µg/ml). *A. remota* and *A. racemosus* had only its methanol extracts showing good activity (IC_{50}=22.1 and 31.35 µg/ml).

S. princeae and *C. edulis* showed no reasonable *in-vitro* activity against D_6 for all the extracts (IC_{50} >100 µg/ml). Apart from chloroform and aqueous extracts of *A. racemosus* that showed no activity, methanol extracts was within the mild activity range.

For *A. remota*, only the methanol extracts showed mild activity of IC_{50}=22.1 µg/ml, whereas the rest were inactive. Methanolic extracts of leaves and rootbark of *C. myricoides* showed good activity against the two isolates used. *C. abyssinica* methanolic extracts for both roots and leaves exhibited reasonable antiplasmodial activity (IC_{50}=6.93 and 26.72 µg/ml). Ajuga methanolic extracts also exhibited reasonable antiplasmodial activity (IC_{50}=22.1 µg/ml).

The *C. myricoides* showed good antiplasmodial activity of the methanolic extract. Although the root bark lacked activity in most of the extracts, the methanolic one gave high activity of IC_{50} = 10.2 µg/ml for D_6 and 9.96 µg/ml for W_2, respectively which is twice as active as that of the leaves IC_{50}=19.96 and 20.58 µg/ml. This is an indication of a better *in vivo* antiplasmodial activity of the root bark than that of the leaves. There are cases in which the chloroquine-resistant isolates seemed to be more responsible to the plant extracts than the susceptible ones. For example, W2 is more than two times susceptible than D_6 to *C. abyssinica* leaves methanolic extracts (IC_{50}=12.22 and 26.72 µg/ml, respectively).

In vivo antiplasmodial activity

Methanol extracts giving optimum antiplasmodial activity *in vitro* were subjected to further investigation in mice for antimalarial potential. Table 3 summarizes the the *in vivo* antimalarial activity of the selected plant extracts.

It was found that *C. abbyssinica, A. racemosus* and *C. myricoides* plants used by traditional healers in Nandi district to treat malaria exhibited good *in vivo* antiplasmodial activity. The methanol extracts are the most active (Tables 1 and 2).

Parasitemia levels are increasing from day 2 to 4 (Table 3). From the chemo suppression data, it shows that parasite clearance was much pronounced on the fourth day.

DISCUSSION

In vitro antiplasmodial assays, *in vivo* antiplasmodial activity and chemo suppression results indicated that methanol extracts were very effective compared to chloroform and water extracts. This would be attributed to the fact that methanol extracts contained many of the bioactive compounds from the plant materials which acts

Table 2. The mean IC_{50} values (X±SD) for selected plants extracts against chloroquine-resistant (W_2) *P. falciparum* isolates.

Extract	IC_{50} (μg/ml)	
	CQ-sensitive isolate (D_6)	CQ-resistant isolate (W_2)
C. myricoides leaves (methanol)	19.96	20.58
Root bark (methanol)	10.2	9.96
C. abyssinica roots (methanol)	6.93	12.22
*Chloroquine (CQ)	0.020	0.069

*Positive control.

Table 3. *In vivo* anti-malarial activity of selected medicinal plant extracts on *Plasmodium berghei* in mice.

Plant	Extract	Parasite density (%)	Chemo suppression (%)
Clutia abbysinica leaves	Methanol	24.03	42.10
Clutia abbysinica roots	Methanol	24.93	39.93
Asparagus racemosus leaves	Methanol	19.07	54.05
Clerodendrum myricoides leaves	Methanol	7.4	82.17
Clerodendrum myricoides root	Methanol	15.83	61.86

on the plasmodia parasites. The major ingredients known to occur in most medicinal plants are the alkaloids, terpenoids and flavonoids, whose presences is attributed to the antimicrobial activity in plants (Nostros et al., 2000). The concentrations and proportions of the active compounds in plant extracts components depend on the plant variety, origin, time of harvest, solvent used, conditions of processing and storage (Deans and Ritchie, 1987). This agrees with the ethnobotanical uses of the plants among the people of Aldai Division of Nandi County (Jeruto et al., 2008).

The methanolic root extract of *C. myricoides* showing good antiplasmodial activity for both D_6 (IC_{50} = 10.2 µg/ml) and W_2 (9.96 µg/ml) is supported by the fact that root bark act as reservoirs for photosynthates or exudates (Balick and Cox, 1996); hence, containing many phytochemicals whose presences is attributed to the antiplasmodial activity in plants. In cases in which the chloroquine resistant isolates seemed to be more responsive to the plant extracts than the susceptible ones, it suggests lack of cross-resistance with chloroquine probably due to differences in the mode of action of compounds present in the extracts. This indicates that the extracts from these plants have the potential of solving the problem of multi-drug resistance. These plants warrant further detailed biological and chemical studies. The bioactive principles could be isolated from these plants and used to develop cheap anti-malarial drugs. *C. myricoides* has also been reported to be useful in the management of other parasitic diseases such as theileriosis (Baerts and Lehman, 1991; Muregi et al., 2004). Phytochemical investigations in the genus *Clerodendrum* has revealed macrocylic alkaloids (Muregi et al., 2004) triterpenoids saponins (Toyota et al., 1990) and iridoid glycosides (Calis et al., 1994) with no indications of their antiplasmodial activity.

Ajuga methanolic extracts exhibiting reasonable antiplasmodial activity would be supported by previous bioassay guided phytochemical investigations that showed cardio tonic (Kuria and Muriuki, 1984) and antiplasmodial activities due to the presence of egosterols-5, 8- peroxide (Kuria et al., 2002).

Lack of *in vitro* antiplasmodial activity on *C. edulis* and *S. princeae* may not necessarily imply the same *in vivo* since compounds may either act as prodrugs, febrifuges (fever is one of the symptoms associated with a complicated severe *P. falciparum* malaria) or immuno-modulators (Muregi, 2004). Active compounds isolated were from the bark of *Melia azedarach* and *Azadirachta indica* which have been found to be antiplasmodial compounds (Khalid et al., 1989). The results showed that the methanol extracts of the species *C. abyssinica*, *A. racemosus* and *C. myricoides* commonly used by traditional healers and the people of Aldai Division of Nandi South District, Nandi County (Jeruto et al., 2008) for the treatment of malaria, exhibited the highest *in vivo* antiplasmodial activity.

Rather low *in vitro* antiplasmodial activity was recorded in the other plants despite the fact that they have been used as traditional antimalarials for centuries. One probable explanation of their popular use in the treatment of malaria could be that they may be useful in managing other manifestations associated with malaria rather than therapeutic (anti-parasitic) activity. These would include reducing fever, convulsions and headache and possibly even immunostimulatory effects (Rasoanaivo et al., 1998).

Several synthetic molecules have been shown to restore CQ-sensitivity in resistant *P. falciparum* (Oduola et al., 1998). Little is known about the mechanism of the reversal of resistance by herbal remedies. Rasoanaivo et al. (1998) have investigated several medicinal plants used in association with chloroquine by local populations in Madagascar. They have shown that not only do the crude alkaloids significantly enhance the *in vitro* and *in vivo* chloroquine action but also the isolated compounds reverse resistance significantly.

The chemo suppression data showed that parasite clearance was much more pronounced on the fourth day. This may be attributed to high drug concentration in the blood due to repeated dosing and the drug has gained access into the parasites and exhibiting its effects.

CONCLUSION AND RECOMMENDATIONS

The fact that the plants screened *in vitro* had some level of antiplasmodial activity would justify their ethno pharmacological uses as traditional antimalarials. The activities observed especially with the methanol extracts indicate that these plants deserve to be investigated further as potential antimalarial agents. It is necessary to carry out detailed phytochemical studies to identify the active constituents in these plants. There are cases where the individual isolated compounds may not inhibit activity unlike their combinations in the crude extracts. This may be explained by synergistic effects of the constituents of the crude extracts or presences of prodrugs. It has been found that some plant extracts may show *in vitro* activity and no *in vivo* activity or vice versa (Gessler et al., 1995). This therefore calls for detailed *in vitro* and *in vivo* investigations of the crude extracts and the isolated compounds. Many communities around the world use these plants as traditional antimalarials (Njoroge and Bussman, 2006). Besides the presence of bioactive compounds, it also depends on many factors such as the season in which the plants is collected, the age of the plants, interspecies variation, part collected, soil and climate among others. Therefore, lack of *in vitro* antiplasmodial activity in this case does not disqualify the use of these plants as traditional antimalarials. All the three plant materials tested for bioassay contained phenolic compounds. It is not yet certain at this point that these phenolics compounds contribute to antimalarial activity. On the other hand, the root extracts contain alkaloids, and the well-known antimalarial, plant origin e.g. quinine, are alkaloids. However, it is worth noting that the plant materials that contain alkaloids also contained terpenoids (Nostros et al., 2000). This is noteworthy because the newer antimalarial compounds, such as quinghaosu, which is already in clinical use, and the quassinoids, are terpenoids.

Further toxicity studies (acute, chronic and cytotoxicity on cells) should be carried out on the crude active fractions to establish their safe levels for use by humans. This should help in calculation of the safety dosage required for parasite clearance. The dosage if calculated would help in improving the safety of *A. racemosus*; *C. myricoides* and *C. abyssinica* based herbal drugs for malarial treatment.

Plants that exhibited moderate *in vivo* antiplasmodial activity in this research need further biochemical investigations. Drug interaction studies to be carried out so that the crude preparation and isolated compounds could be used in combination with chloroquine against resistant strains of *P. falciparum*.

Conflict of interest

The authors declare no conflict of interest.

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