



In vivo antimalarial activity, toxicity and phytochemical screening of selected antimalarial plants

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ABSTRACT

Ethnopharmacological relevance: Malaria continues to kill over a million people each year and in many populations affected by malaria, conventional drugs are often unaffordable or inaccessible. Historically, plants have been a prominent source of antimalarial drugs. Those plants currently used by indigenous people to treat malaria should be documented and investigated as potential sources of new antimalarial drugs.

Aim of the study: To investigate *in vivo* antimalarial activity, toxicity and carry out phytochemical screening of selected plants which have been used in traditional medicine for the treatment of malaria.

Materials and methods: Organic and water extracts of four medicinal plants used for the treatment of malaria in traditional health systems of Msambweni people in Kenya were tested for antimalarial activity against *Plasmodium berghei* and brine shrimp lethality. They were also screened for their major phytochemical constituents.

Results: Aqueous extract of the stem bark of *Adansonia digitata* exhibited highest chemosuppression of parasitaemia, > 60% in a murine model of *Plasmodium berghei* infected mice. Aqueous and organic extracts of *Launaea cornuta* and *Zanthoxylum chalybeum* were toxic to the brine shrimp ($LD_{50} < 1000 \mu\text{g/ml}$) while aqueous and organic extracts of *Adansonia digitata* and aqueous extracts of *Canthium glaucum* were not toxic to brine shrimp ($LD_{50} > 1000 \mu\text{g/ml}$). Phytochemical screening revealed the presence of alkaloids and flavonoids in all the crude extracts of the selected plant species studied. Sesquiterpene lactones and saponins were present in organic extracts but absent in the aqueous extracts of *Adansonia digitata*, *Canthium glaucum*, *Launaea cornuta* and *Zanthoxylum chalybeum*.

Conclusion: The results showed that the crude extracts of *Adansonia digitata* and *Canthium glaucum* demonstrated promising antimalarial activity and there is potential for isolation of lead compounds from their extracts.

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1. Introduction

Malaria continues to kill over a million people each year, with more than 90% of these cases found in sub-Saharan Africa (Nguta et al., 2010). In many populations affected by malaria, conventional drugs are often unaffordable or inaccessible, and increasing drug resistance by the malaria parasite, *Plasmodium falciparum* is of significant concern (Greenwood and Mutabingwa, 2002). As an alternative, medicinal plants are often used to treat malaria. Historically, plants have been a prominent source of antimalarial drugs like quinine and artemisinin (Saxena et al., 2003). Moreover, those plants currently used by indigenous people to treat malaria should be documented and investigated as potential sources of new antimalarial drugs. This study investigated the antimalarial activity, toxicity and the nature of major chemical

classes present in four medicinal plants: *Launaea cornuta* (Hocht. ex.Oliv. and Hiern.) C. Jeffrey (Compositae), *Zanthoxylum chalybeum* Engl. (Rutaceae), *Adansonia digitata* L. (Bombacaceae) and *Canthium glaucum* Hiern. (Rubiaceae) used by the Msambweni community of South Coast Kenya for treatment of malaria or malaria like symptoms.

2. Materials and methods

2.1. Plant materials

The four plant samples used in this study were collected between May and November, 2009 from Msambweni district of Kenya. They were selected based on the ethnopharmacological use as antimalarial through interviews with local communities (Nguta et al., 2010). Information gathered included vernacular names (in parentheses) and the parts used in preparation of the herbal antimalarial remedies:

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Adansonia digitata (Mbamburi) stem bark, *Canthium glaucum* (Mhonga) roots, *Launaea cornuta* (Mtsunga wa utsunga) leaves and *Zanthoxylum chalybeum* (Mjafari/mporojo) stem bark. The plants were identified by taxonomists at the East African Herbarium, National Museums of Kenya, Nairobi, where voucher specimens were deposited. Voucher numbers (in parentheses) of the collected plants are: *Adansonia digitata* (JN414), *Canthium glaucum* (JN426), *Launaea cornuta* (JN028) and *Zanthoxylum chalybeum* (JN040). Species nomenclature follows the flora for tropical East Africa. A written informed consent was obtained from all the respondents in the study. The plant parts were chopped into small pieces, air dried at room temperature (25 °C) under shade and pulverized using a laboratory mill.

2.2. Preparation of crude extracts

Organic extracts were obtained by extraction of, 50 g of powdered material of each of the selected plants separately in 500 ml of methanol:chloroform (1:1) four times at 24 h intervals. Each of the extracts was filtered and then concentrated individually under reduced pressure at temperature below 50 °C to obtain the crude extracts of the samples. Aqueous hot infusion of each plant part was prepared (50 g of powdered material in 500 ml of distilled water) in a water bath at 60 °C for 1 h. Filtrate from each plant was kept in a deep freezer then lyophilized resulting to a dry powder. The yields of the water extracts were higher than the corresponding organic extracts and ranged between 4.5% and 12.8%, while those of the organic extracts were between 4.1% and 8.4%. The dry solid extracts were stored at –20 °C in airtight containers.

2.2.1. Sample stock solutions

A stock solution of each of the aqueous crude extracts (10,000 µg/ml) was prepared by dissolving 0.1 g of each extract in 10 ml of distilled water. The same concentration of the organic extracts was prepared by separately dissolving, 0.1 g of each of the samples in dimethylsulfoxide (DMSO) and diluted the mixture using artificial water to 10 ml. The stock solutions from various crude extracts were filtered using 0.22 µm micro-filters under lamina flow hood (Wanyoike et al., 2004).

2.3. Toxicity testing against the brine shrimp

2.3.1. Hatching shrimp

Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water prepared by dissolving 33 g of sea salt (Sigma chemicals Co., UK) in 1 l distilled water. Incubation was done at room temperature (22–29 °C) for 48 h after which the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with a pipette. Brine shrimp larvae were separated from eggs by aliquoting them three times in small beakers containing sea water.

2.3.2. Brine shrimp assay

The toxicity of the extracts was monitored by the brine shrimp lethality test (Meyer et al., 1982; Wanyoike et al., 2004). Using Pasteur pipettes, 10 brine shrimp larvae were transferred from the smaller compartment of the hatching tank to each of the plastic tubes. The volume of artificial sea water in each plastic tube was increased to 5 ml except the tubes containing 1000 µg/ml which were topped up to 4.5 ml with artificial sea water. Using micropipettes, 0.5, 0.05 and 0.005 ml were transferred from the stock solutions to the individual plastic tubes containing 5 ml artificial sea water to make experimental solutions containing 1000, 100 and 10 µg/ml respectively. A control group containing artificial sea water and brine shrimp larvae only was included in the experiment. Each experiment was carried out in triplicate and

the survivors were counted after 24 h using a magnifying glass (Meyer et al., 1982).

2.4. In vivo determination of antimalarial activity

In vivo antimalarial activity was determined by a 4-day suppressive antimalarial assay according to Waako et al. (2005). Cryopreserved chloroquine sensitive *Plasmodium berghei* (ANKA strain) were first revived and stabilized in mouse host according to Ravindran et al. (1982) before they were introduced to experimental mice. Once the *Plasmodium berghei* parasites had stabilized in the mouse host, one mouse was chosen as a donor mouse which was then anesthetized using chloroform in a fume chamber and blood collected via cardiac puncture into heparinized bottles to make inoculum for infecting experimental mice. Appropriate inoculum for infecting mice should have low parasitaemia of around 1% (Waako et al., 2005) and experimental mice were inoculated with 0.2 ml of blood with 1% parasitaemia (containing about 1.0×10^7 parasitized cells). Swiss albino mice [*Mus musculus* L. (Muridae)] about 8 weeks old weighing between 18 and 22 g, irrespective of sex were then infected with the *Plasmodium berghei* parasites and kept in the main chamber before they were divided into groups of five.

A completely randomized design was employed in conducting the experiment. Ten cages of mice were selected and in each cage, 5 infected mice were assigned randomly from the main chamber. The cages were assigned treatments randomly. Mice in four cages received oral administration of aqueous extracts at a dosage of 100 mg/kg/day for 4 days. Another group of mice in four cages received oral administration of CHCl₃:MeOH extracts at a dosage of 100 mg/kg/day for 4 days. The last two cages were used as control cages; one positive control and one negative control. Plant extracts were administered once daily orally (Days 0–3) at a dosage of 100 mg/kg (2 mg/mouse/day) in a dose volume of 0.2 ml. Positive control drug used was chloroquine 20 mg/kg/day (0.4 mg/mouse/day) while negative control group was treated with distilled water (0.2 mls/mouse/day). On the first day, plant extracts were administered 2 h after infection of the mice with the parasites.

Blood was obtained from each of the experimental mice on Day 4 via a tail cut from which thin blood smears were prepared. Smears were fixed with methanol for 5 min and stained with 10% Geimsa. The slides were observed under compound microscope under oil immersion at $\times 1000$ to determine the number of parasitized cell per given magnification field. For each blood smear specific for a given mouse, four magnification fields were observed and the number of parasitized cells and the total number of cells in the magnification field were recorded. The data obtained was used to determine percentage parasitaemia and percentage chemosuppression in each mouse. The number of dead mice were also recorded every day since the start of the 4 day suppressive test for the next 10 days.

2.5. Phytochemical screening for secondary metabolites

Crude plant extracts were screened for alkaloids, flavonoids, saponins and sesquiterpene lactones on Thin Layer Chromatography (TLC) plates using appropriate spray reagents and UV absorbance according to procedures described by Harborne (2002).

2.6. Statistical analysis

Chemosuppression was analyzed using SPSS Version 16. One way ANOVA was used to analyze chemosuppression means obtained from the 4 day suppressive assay to determine whether chemosuppression caused by one plant extract was different from chemosuppressions caused by the other plants extracts according to Morgan et al. (2004).

In cases where the means were found to be different from each other, the Dunnett test was used for multiple comparisons of chemosuppressions to determine whether chemosuppressions arising from the various treatments were different from the chemosuppression induced by chloroquine (positive control). The significance level used in the analysis was 0.05 (Alpha level < 0.05). Acute toxicity (LD₅₀) of the crude extracts on brine shrimp larvae was estimated using the Finney probit analysis.

3. Results

3.1. Acute toxicity of the crude extracts

The numbers of dead and surviving brine shrimp larvae were recorded 24 h after they were subjected to various concentrations of aqueous and organic extracts of four plants. The average mortality in the three concentrations (1000, 100 and 10 µg/ml) for each plant extract were subjected to Finney's probit analysis to estimate the LD₅₀ according to Meyer et al. (1982). The results are summarized in Table 1.

Plant extracts with LD₅₀ values ranging between 0 and 500 µg/ml imply highly toxic, 500 and 1000 µg/ml moderately toxic and over 1000 µg/ml nontoxic (Nguta et al., 2011). Results showed that extracts of *Adansonia digitata* stem bark and *Canthium glaucum* roots were non toxic to brine shrimp larvae. However extracts of *Zanthoxylum chalybeum* stem bark and *Launaea cornuta* leaves were considered toxic to brine shrimp larvae.

3.2. In vivo antimalarial activity

The total number of parasitized cells and the total number of cells in four magnification fields in each microscope slide were used to determine parasitaemia and chemosuppression in each mouse. Percentage parasitaemia and percentage chemosuppression were determined using the formulae described by Hilou et al. (2006) respectively and summarized in Table 2.

$$\% \text{Parasitaemia} = (\text{Total number of parasitized cells} / \text{Total number of cells}) \times 100\%$$

$$\text{Chemosuppression} = [(\text{Negative control parasitaemia}) - (\text{Parasitaemia with drug})] / \text{Negative control parasitaemia}$$

From Table 2, plant extracts that induced high to moderate chemosuppression were the aqueous extracts of *Adansonia digitata*, *Launaea cornuta*, *Zanthoxylum chalybeum* and organic extract of *Canthium glaucum*. The antimalarial drug chloroquine (positive control) which was used in the experiment induced highest chemosuppression.

Mortality of the mice were monitored on daily basis. Each of the groups treated with organic extracts of *Canthium glaucum* and aqueous extracts of *Adansonia digitata*, *Canthium glaucum* and *Zanthoxylum chalybeum* had one mouse alive on the 10th day. No mouse died in the positive control group which received oral administration of chloroquine on daily basis and all the 5 mice were healthy on the 10th day. In the negative control group which was receiving distilled water on daily basis, all the 5 mice were 7th day post infection.

3.3. Phytochemical analysis

See Table 3.

4. Discussion

The percentage parasitaemia in the negative control group on Day 4 after infection with *Plasmodium berghei* parasites was 31.88% which was the highest observed. Lowest parasitaemia (4.07%) was observed in the group which was treated with chloroquine. Parasitaemia in the negative control was higher than in all the treatment groups. This showed that all the treatments had effect on the growth of *Plasmodium berghei* parasites in mice. Parasitaemia increased gradually in all the groups, and all the mice died on the 7th day in the negative control group. All the

Table 1
Acute toxicity of the crude plant extracts.

Plant name	Average mortality at various concentrations (µg/ml)								LD ₅₀ (µg/ml)	
	1000		100		10		0		Aq	Org
	Aq	Org	Aq	Org	Aq	Org	Aq	Org		
<i>Adansonia digitata</i> L.	2.33	10	0.33	3.33	0	0.66	0	0	9563.59	110.23
<i>Canthium glaucum</i> Hiern.	5.33	2	0.33	0.66	0.66	0	0	0	1209.67	1783.59
<i>Launaea cornuta</i> (Hocht.ex.Oliv.) C.Jeffrey	8	10	1.33	4	0.33	2	0	0	341.64	75.26
<i>Zanthoxylum chalybeum</i> Engl.	10	10	0.33	9	0	2	0	0	268.28	25.78

Aq=aqueous extract, Org=organic extract.

Table 2
Mean parasite density, chemosuppression and survival time of *Plasmodium berghei* infected mice.

Plant name	Mean (X ± SEM) parasite density (%)		Mean (X ± SEM) chemosuppression (%)		Mice survival by day 10 (%)	
	Aq	Org	Aq	Org	Aq	Org
<i>Adansonia digitata</i> L.	12.60 ± 5.11	21.39 ± 4.59	60.47 ± 16.03	32.90 ± 14.40	20	0
<i>Canthium glaucum</i> Hiern.	21.68 ± 2.42	17.92 ± 2.49	31.98 ± 7.60	43.76 ± 7.83	20	20
<i>Launaea cornuta</i> (Hocht.ex.Oliv.) C.Jeffrey	19.72 ± 0.99	21.98 ± 5.21	38.13 ± 3.13	31.04 ± 16.33	0	0
<i>Zanthoxylum chalybeum</i> Engl.	17.56 ± 3.62	23.09 ± 1.16	44.93 ± 11.36	27.56 ± 3.635	20	0
Negative control	31.88 ± 1.11		0		0	
Positive control	4.07 ± 0.81		87.23 ± 2.72		100	

P=0.05, SEM=standard error of the mean, Aq=aqueous extract, Org=organic extract.

Table 3
Phytochemical screening of aqueous and CHCl₃: MeOH crude extracts.

Plant name	Alkaloids		Flavonoids		Sesquiterpene lactones		Saponins	
	Aq	Org	Aq	Org	Aq	Org	Aq	Org
<i>Adansoniadigitata</i> L.	+ve	+ve	+ve	+ve	–ve	+ve	–ve	+ve
<i>Canthium glaucum</i> Hiern.	+ve	+ve	+ve	+ve	–ve	+ve	–ve	+ve
<i>Launaea cornuta</i> (Hocht.ex.Oliv.)C.Jeffrey	+ve	+ve	+ve	+ve	–ve	+ve	+ve	+ve
<i>Zanthoxylum chalybeum</i> .Engl.	+ve	+ve	+ve	+ve	–ve	+ve	–ve	+ve

+ve=Present, –ve=absent, Aq=aqueous extract, Org=organic extract.

mice were alive and healthy on the 10th day in the positive control group.

Drugs lead to decreased parasitaemia and subsequent recovery of symptomatic malaria. They also reduce parasitaemia through various ways like reducing parasite nutrient intake, interfering with parasite metabolic pathways like heme metabolic pathway which is involved in the metabolism of iron (Devillers and Egon, 2009). Drugs also negatively affect parasite reproduction and growth (Ziegler et al., 2002). The plant extracts reduced the level of parasitaemia and made some of the mice to survive up to the 10th day. Chloroquine had a good chemosuppression of 87.23% as determined on the fourth day post-infection and a 100% survival rate by 10th day post-infection.

Chemosuppression is inversely related to parasitaemia. In this study, plant extracts which have been shown to reduce parasitaemia to low levels demonstrated corresponding high chemosuppression. The aqueous extract of *Adansonia digitata* had a chemosuppression of 60.47% ($p < 0.05$), which was the highest chemosuppression recorded in the experiment from all the plant extracts tested. Other extracts which had moderately high chemosuppression included the organic extract of *Canthium glaucum*, aqueous extract of *Zanthoxylum chalybeum* and aqueous extract of *Launaea cornuta* with 43.76%, 44.93% and 38.13% respectively.

Aqueous extracts of *Adansonia digitata* induced a chemosuppression which was not significantly different from that induced by chloroquine ($P < 0.05$). Although the other plant extracts induced chemosuppression which was significantly different than that induced by chloroquine, to some extent they inhibited the growth of *P. berghei* parasites. By 10th day post-infection, each of the groups treated with organic extract of *Canthium glaucum*, aqueous extracts of *Canthium glaucum* and *Zanthoxylum chalybeum* had one mouse alive indicating that the plant extracts had *in vivo* antimalarial activity though not matching that of chloroquine.

From the study, crude extract of the stem bark of *Adansonia digitata* exhibited high antimalarial activity. This observation supports earlier work by Ajaiyeoba (2005) who reported that methanolic extracts of the stem bark of *Adansonia digitata* were able to reduce the number of *Plasmodium* parasites in mice. Previous studies have shown that water and methanol extracts of the stem bark of *Zanthoxylum chalybeum* have significant *in vitro* antimalarial activity against chloroquine sensitive and chloroquine resistance strains of *P. falciparum* (Rukunga et al., 2009) which is in line with the results on *Zanthoxylum chalybeum* obtained in the current study. Methanolic extracts of the root bark of *Zanthoxylum chalybeum* have been reported to have significant antimalarial activity on *P. falciparum* (Muganga et al., 2010). This is also in agreement with the observations in the current study. In other studies, aqueous extract of a related species *Zanthoxylum usambarense* (Engl.) also exhibited significant antiplasmodial activity against *P. falciparum* (Kirira et al., 2006).

Aqueous extracts of both *Adansonia digitata* and *Canthium glaucum* had a LD₅₀ > 1000 µg/ml, hence, were considered to be non toxic to brine shrimp larvae while aqueous and organic extracts of *Launaea*

cornuta, *Zanthoxylum chalybeum* with a LD₅₀ < 500 µg/ml were considered toxic to brine shrimp larvae. A Previous study by Ramadan et al. (1994) reported that aqueous extract of the fruit pulp of *Adansonia digitata* had a LD₅₀ > 8000 µg/ml and was categorized as being non toxic to mice. This supports the results obtained in this study on the nontoxic nature of stem bark of *Adansonia digitata* on brine shrimp larvae. Non toxicity of *Adansonia digitata* explains why most of the plant parts: seeds, fruit pulps and leaves are consumed by many communities (Kamatou et al., 2011). Nguta et al. (2011) while investigating toxicity of aqueous extracts of the leaves, stem bark and root bark of *Zanthoxylum chalybeum* on brine shrimp larvae obtained LD₅₀ < 500 µg/ml for the three plant parts which supports the results obtained in the current study indicating that *Zanthoxylum chalybeum* could not make safe antimalarial remedies. This calls for dose adjustment amongst communities that use this plant for preparation of antimalarial herbal decoctions. In herbal practice, *Zanthoxylum chalybeum* is usually prepared as a concoction with other antimalarial plants (Nguta et al., 2010), and this could explain why no adverse effects have been reported by those communities commonly using the plant as an antimalarial phytotherapeutic remedy. Significant cytotoxicity of *Zanthoxylum chalybeum* methanolic root bark extract on human normal fetal lung fibroblast cells has also been reported by Kamuhabwa et al. (2000). Similarly high cytotoxicity of stem bark of *Launaea cornuta* on brine shrimp larvae was reported by Nguta et al. (2011) and high toxicity of the crude leaves extract of *Launaea cornuta* on brine shrimp larvae witnessed in this study are in agreement with observations from the previous study.

Non toxicity of *Adansonia digitata* and *Canthium glaucum* extracts suggest that the extracts from these plants have a potential to inhibit the growth of *Plasmodium* parasites which is not associated with their inherent toxicity. For instance, aqueous extract of *Zanthoxylum chalybeum* had a slightly high chemosuppression of 44.93% and were highly toxic (with a LD₅₀ of 268.28 µg/ml) on brine shrimp larvae implying that the toxicity of the extracts could have lead to observed chemosuppression of the *Plasmodium berghei* parasites.

All the plant extracts used in the experiment were screened for the presence or the absence of various classes of secondary metabolites using Thin Layer Chromatography. Alkaloids and flavonoids were found to be present in all crude plant extracts. Sesquiterpene lactones were present in the organic extracts of *Adansonia digitata*, *Canthium glaucum*, *Launaea cornuta* and *Zanthoxylum chalybeum*. Saponins were present in organic extracts of *Adansonia digitata*, *Canthium glaucum*, *Launaea cornuta* and *Zanthoxylum chalybeum* and aqueous extracts of *Launaea cornuta*. Alkaloids, flavonoids and sesquiterpenes have been reported to be potent plant secondary metabolites with broad spectrum of bioactivities (Mazid et al., 2011). Alkaloids are major classes of compounds possessing antimalarial activity; quinine is one of the most important and oldest antimalarial drugs which belongs to this class of compounds (Saxena et al., 2003).

Biological activity is attributed to the presence of various secondary metabolites in plants (Mazid et al., 2011). In view of this, it is envisaged that any one of the classes of compounds may

be responsible for the activity. Alkaloids and flavonoids in aqueous stem bark extract of *Adansonia digitata* and alkaloids, flavonoids, saponins, sesquiterpene lactones in organic extract of *Canthium glaucum* may be the active constituents responsible for the observed *in vivo* antimalarial activity. Cytotoxicity is also attributed to the presence of various secondary metabolites found in plant extracts. Not only their presence, but also the quantity of the phytochemical constituents in a given plant extract will determine the extent of its bioactivity. In addition, presence of more than one class of secondary metabolites in a given plant extract determines the nature and extent of its biological activity (Wang et al., 2010). Hence the various chemical compounds may be present in high concentration in aqueous extract of *Adansonia digitata* and organic extract of *Canthium glaucum* and this may be responsible for their high antimalarial activity.

5. Conclusion

This study has showed that organic extract of *Canthium glaucum* and aqueous extract of *Adansonia digitata* significantly inhibited the growth of *P. berghei* parasites. Indeed, aqueous extract of *Adansonia digitata* had a chemosuppression of 60.47% an indication of good antimalarial activity against *P. berghei* parasites. In terms of toxicity, the stem bark of *Adansonia digitata* and roots of *Canthium glaucum* were non toxic to the *A. salina* larvae. The study reports for the first time the *in vivo* antimalarial activity of *Canthium glaucum* and *Launaea cornuta*. In addition this study validates the anecdotal efficacy of antimalarial activities of *Adansonia digitata* and *Zanthoxylum chalybeum* reported from traditional medicine of a Kenyan coastal community. Further studies using bioassay guided fractionation of crude extracts of *Adansonia digitata* and *Canthium glaucum* are needed to isolate and identify the active compounds. Their active constituents may be potential candidates with therapeutic value in the treatment of malaria.

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