Vangueria infausta root bark: *in vivo* and *in vitro* antiplasmodial activity

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ABSTRACT

Vangueria infausta burch subsp. *infausta* (Rubiaceae) produces fruits eaten by humans and animals. The leaf, fruit, stem bark and root bark are used as a remedy for many ailments and the roots are used to treat malaria. In this study, concentrations of fractions of the *V. infausta* root bark extract that produce 50% inhibition (IC_{50}) are determined using the ability of the extract to inhibit the uptake of [G^3H]-hypoxanthine by *P. falciparum* cultured *in vitro*. The root bark extract showed antimalarial activity against *Plasmodium berghei* in mice. It gave a parasite suppression of 73.5% in early infection and a repository effect of 88.7%. One fraction obtained from a chloroform extract gave an IC_{50} value of 3.8±1.5 µg/mL and 4.5±2.3 µg/mL against D_6 and W_2 strains of *P. falciparum*, respectively, and another from the butanol extract gave an IC_{50} value of 3.9±0.3 µg/mL against the D6 strain. Chloroquine had an IC_{50} value of 0.016 µg/mL and 0.029 µg/mL against D_6 and W_2 strains, respectively. The plant showed the presence of flavonoids, coumarins, tannins, terpenoids, anthraquinones and saponins.

KEY WORDS: Biological factors.
Plant extracts. Plants, medicinal.
Plasmodium berghei.
Plasmodium falciparum.
Vangueria infausta.

Introduction

Malaria affects a large number of people in tropical and subtropical areas of the world. The majority of the deaths caused by the disease occur in Africa, a continent that also carries the burden of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS).[1] In addition, control of the disease is often disturbed by civil unrest, which is commonplace in this part of the world.

The resurgence of malaria, vector resistance to insecticides and resistance of *Plasmodium* species to the most valuable chemotherapeutic agents such as chloroquine[2–6] (Fig. 1) has magnified the problem, which means that malaria remains one of the world's most common tropical diseases.

The success of artemisinin (Fig. 2), a sesquiterpene lactone with endoperoxide moiety, isolated from a traditional Chinese medicinal plant[7] opened a new horizon in antimalarial drug research and increased interest in plants with medicinal properties. Thus, there is hope that secondary metabolites with specific actions against malaria might be isolated.

In many tropical countries, a wide variety of reputable medicinal plants[8–11] are sold by local medicinal herb vendors for treatment of a variety of diseases. Research work carried out on marketed medicinal plants in Botswana shows that most of these plants contain secondary metabolites with medicinal properties, some of which have antiplasmodial activity.[12]

Vangueria infausta burch subsp. *infausta* (Rubiaceae) produces fruits eaten by humans and animals. The leaf, fruit, stem bark and root bark are used as a remedy for many ailments and the root is used to treat malaria.[13–15] Nundkumar and Ojewole[16] demonstrated antimalarial activity of the leaf extract of this plant.

Previous research shows that the leaf, fruit, stem bark and root bark possess antimalarial activity.[17] The present study reports the antimalarial activity of the crude root bark extract and the concentrations of fractions obtained that produce 50% inhibition (IC_{50}) of the *Plasmodium* species.

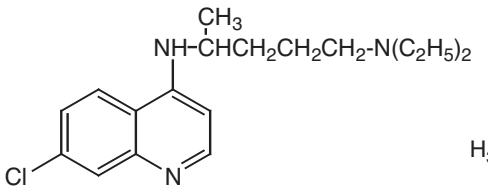

Fig. 1. Chemical structure of chloroquine, to which *Plasmodium* species are becoming increasingly resistant.

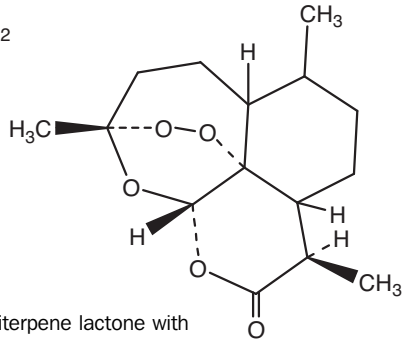

Fig. 2. Chemical structure of artemisinin, a sesquiterpene lactone with endoperoxide moiety, isolated from a traditional Chinese medicinal plant.

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Materials and methods

Plant material

The root bark of *V. infausta* was collected from Veld Product Farms, Gabane, and from Mapoka in north-east Botswana in March and June 2003, respectively.

General methodology

Chromatography was carried out using silica gel 60 with a particle size of 0.040–0.063 mm for column chromatography (Merck), silica gel 60 $PF_{254+366}$ for preparative thin-layer chromatography (TLC, Merck) and Sephadex LH-20 for gel filtration. Analytical TLC was performed on TLC silica gel 60-F_{254} precoated alumina sheets (Merck), visualised using ultraviolet (UV) light (254 and 366 nm) and sprayed with vanillin sulphuric acid spray.

Extraction

The dried and powdered root bark (29.3 g) extracted in methanol by the Soxhlet extraction method[18,19] was used in the *in vivo* evaluation. The dried crude extract was redissolved in ethanol and then reconstituted in distilled water to concentrations of 500, 250 and 125 mg/kg prior to use. Each dose was given as a 0.5 mL volume per mouse.

For the *in vitro* antiplasmodial activity evaluation, the dried powdered root bark (600 g) was extracted (x5) in a mixture of solvents (hexane/chloroform/methanol/water, 1:14:4:1) to yield a crude extract that was concentrated under reduced pressure. The dark brown crude extract (59.9 g) obtained was subjected to liquid–liquid partitioning to yield a hexane extract (0.7 g), a chloroform extract (5.3 g), a butanol extract (13.7 g) and residual water extract (16.0 g).

The chloroform extract was subjected to column chromatography (CC) using silica gel (60 g) and eluted with 100% *n*-hexane followed by *n*-hexane/chloroform (1:1), chloroform/ethyl acetate and ethyl acetate/methanol in increasing polarity. A total of 39 fractions collected and examined by TLC (solvent system: *n*-hexane/$CHCl_3$ [9:1, 6:4, 3:7] and $CHCl_3$/EtOAc [9:1]) were then pooled according to similarity of their TLC profiles.

The butanol extract was subjected to vacuum liquid chromatography (VLC) and eluted with chloroform and methanol in increasing polarity to yield six major fractions. The eluting solvents were eliminated by vacuum evaporation. The fractionation scheme is shown in Figure 3.

Phytochemical screening methods

Phytochemical screening was performed according to the method of Chhabra *et al*.[20] This method uses chemical reagents to demonstrate classes of phytochemical compounds present in plant materials.

In vivo *antimalarial activity*

In vivo antimalarial activity was evaluated in both early and established infection, as described by Abosi and Raseroka.[21] To assess any possible repository effect of the extract, a modification of the method by Peters[22] was used. In this method, 25 NMRI albino mice weighing 18–22 g and kept in groups of five in plastic cages at 20°C (room temperature) were used. They were supplied with dog feed and drinking water, allowed free movement and kept in similar environmental condition throughout the experiment.

Each group of mice was given an oral dose of extract on three consecutive days. A dose of 1.2 mg/kg per day of pyrimethamine, a prophylactic drug, was used as the standard drug. This was given to one group while another group received sterile distilled water.

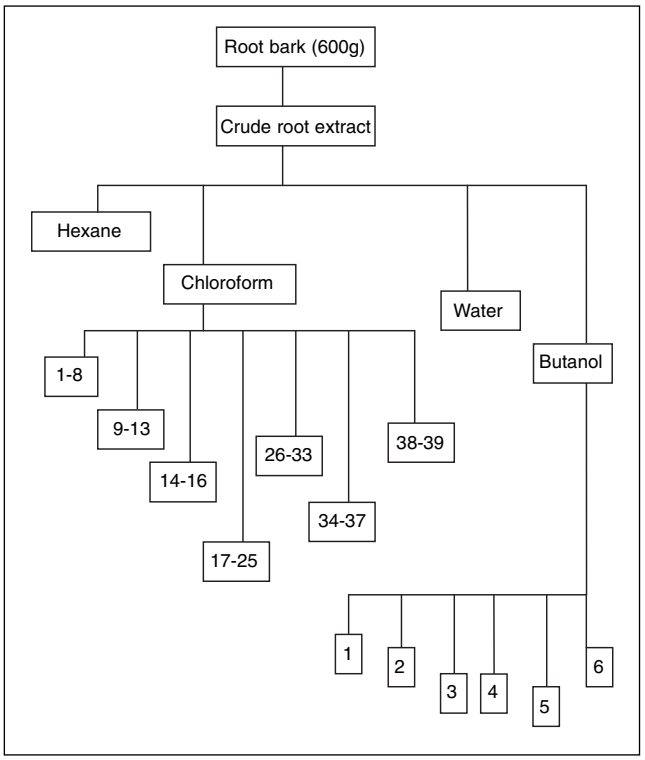

Fig. 3. Fractionation of *V. infausta* for *in vitro* antimalarial testing.

On the fourth day of treatment, the mice were injected with a 1 x 10^7 inoculum of *P. berghei*-parasitised erythrocytes from a donor mouse previously infected with *P. berghei* parasites. Seventy-two hours later, tail-blood smears were prepared, stained by Giemsa and the percentage parasitaemia was determined and compared to controls. The mean percentage suppression was calculated as in early infection.[21]

In vitro *antiplasmodial activity*

Two strains of *P. falciparum*, obtained from the United States Army Medical Research Unit–Kenya (MRU) and maintained in continuous culture at 37°C in a mixture of gases (3% O_2, 6% CO_2 and 91% N_2), were used to assess the antiplasmodial activity of the extracts using a method adapted from that of Desjardin *et al*.[23]

The chloroquine-sensitive Sierra Leone 1 (D_6) and chloroquine-resistant Indo-China 1 (W_2) strains, commonly used in drug sensitivity assays, were grown in a 6% suspension of human AB Positive erythrocytes in culture medium, using a modification proposed by Trager and Jensen,[24] and Haynes.[25]

Once a parasitaemia of 3%, with at least 70% ring forms, was attained the cultures were diluted to a haematocrit of 1% and parasitaemia of 0.9% with non-infected blood. The dried extracts were initially dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 1 mg/mL and serially diluted with culture medium to provide a range of concentrations used to determine IC_{50} values accurately.

For more accurate determination of IC_{50} values of the extracts, 12 two-fold dilutions were prepared starting at

50 µg/mL. Aliquots (25 µL) of the diluted extract were dispensed into 96-well microtitre plates, and 200 µL diluted parasites were transferred to each well.

Two series of controls were included: one with parasitised blood without extract and another with uninfected blood. Standard drugs were also included.

The plates were incubated at 37°C in a CO_2 incubator in an atmosphere of 6% CO_2 for 24 h. The plates were then removed from the incubator and 25 µL isotope in culture medium was added to each well. The isotope was previously prepared to contain 20 µCi [G^3H]-hypoxanthine per mL culture medium.

The plates were returned to the 37°C incubator for an additional 18 hours. They were then moved to –20°C freezer for a further 12 hours. This terminated the assay by stopping parasite growth and ensured complete lysis of the erythrocytes.

Cells were harvested on a glass-fibre filter (Packard Filtermate Harvester Unifilter-96) using an automated cell harvester (Filtermate Cell Harvester, Packard Instruments), washed thoroughly with distilled water to eliminate unincorporated isotope and then allowed to dry.

Using a liquid scintillation counter (Packard Topcount M&T microplate scintillation and luminescence counter), raw data on parasite counts were acquired. These were received in Microsoft Excel spreadsheet format, saved on a diskette and were imported to the data analysis software (Oracle), which gave the result as IC_{50} values for the tested extracts.

Results

In vivo *antimalarial activity*

In a well-established infection (Fig. 4), the mice achieved a range of parasitaemia levels between 8.0% and 14.6% in 72 h following *P. berghei* passage. The different doses of the extract reduced parasitaemia but could not completely eliminate it. There was a gradual daily increase in parasitaemia, with only a slight reduction in parasite count after three days of treatment.

The control group showed daily rises in parasite count, while in the chloroquine-treated group the parasites were almost completely eliminated. Mean survival times of 14.2±0.7 days, 8.8±0.8 days and 7.8±0.8 days were recorded for 500 mg/kg, 250 mg/kg and 125 mg/kg, respectively.

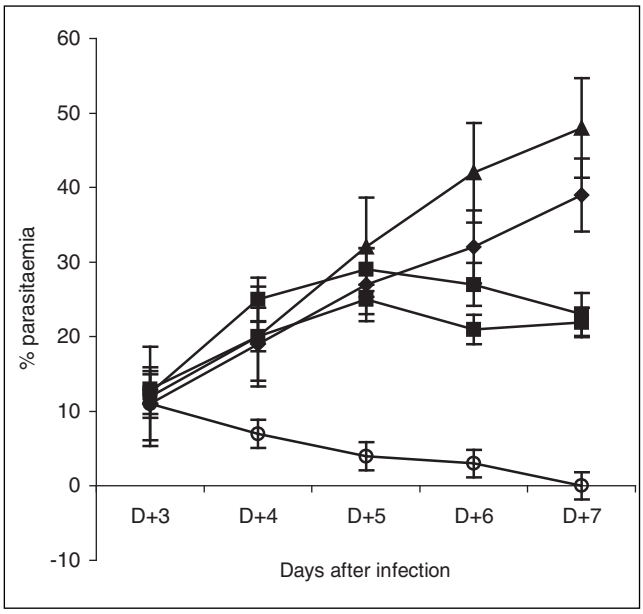

Fig. 4. The effects of *V. infausta* on the level of parasitaemia in mice infected by *P. berghei*. Root bark extract given at 500mg/kg per day; ■—■ 250 mg/kg per day; ◆—◆ 125 mg/kg per day. O—O chloroquine, ▲—▲ control. Each point represents the mean±SE of results from five mice.

The control group showed a mean survival time of 7.6±0.6 days, while the chloroquine-treated group survived for more than 30 days. In early infection and in the repository state (Table 1), parasite suppression was observed and the extracts showed residual effect at higher concentrations, suggesting a dose-related suppressive effect.

In vitro *antiplasmodial activity*

The results of the *in vitro* antiplasmodial evaluation of fractions of *V. infausta* against chloroquine-sensitive D_6 strains and chloroquine-resistant W_2 strains of

Table 1. The *in vivo* antimalarial activity of *V. infausta* root extracts on *P. berghei* in mice.

		Early infection		Repository effect	
	Dose (mg/kg)	Parasitaemia*	Suppression†	Parasitaemia‡	Suppression§
Root	500	10.6±3.9	73.5	2.6±1.8	88.7
Root	250	13.0±1.5	67.5	3.2±1.8	86
Root	125	22.2±2.7	44.5	20.6±2.1	10.4
Chloroquine	5	1.8±0.4	80.4	ND	ND
Control	0	40.0±0.9	0	ND	ND
Pyrimethamine	1.2	ND	ND	2.2±0.2	90.4
Control	ND	ND	ND	23.0±2.3	0

*Mean±SE percentage parasitaemia in early infection.

†Mean parasite suppression in early infection.

‡Mean±SE percentage parasitaemia in repository effect of extract.

§Mean parasite suppression during repository effect.

ND: Not done.

P. falciparum, based on the inhibition of [G^{3}H]-hypoxanthine uptake.

Table 2 displays the antiplasmodial activity of the five main crude extracts, all of which showed antiplasmodial activity. Hexane extract showed the strongest activity against D6 strain.

Table 3 shows that stronger activity was apparent, as the extracts were semipurified. Two fractions gave IC_{50} values <5 μg/mL, five had IC_{50} values <10 μg/mL, while the majority had values <50 μg/mL.

Phytochemical screening

Preliminary phytochemical screening tests showed the presence of flavonoids, coumarins, tannins, terpenoids/steroids and anthraquinones in the root bark extracts.

Discussion

The *in vivo* activity of *V. infausta* root bark extracts against malaria was tested to assess its ability to protect mice from the lethal effects of *P. berghei* infection. The effects produced show that the root extract reduced the level of parasitaemia in early infection, had a residual effect and also influenced the course of well-established infection in mice. The extract-treated mice survived longer than those in the control group.

The results of this *in vivo* evaluation probably explains why this plant is used by the indigenous people[14] and suggests that its use probably reduces levels of parasitaemia such that infection might be symptomless. However, effectiveness might also be influenced by differences in gastrointestinal uptake of the active substances.

Solvent fractionation of the extract aimed to obtain major fractions for antiplasmodial testing by sequentially extracting in hexane, chloroform, butanol and also using the residual water fraction. Assuming that antimalarial activity would result from more polar substances, chloroform and butanol extracts were further fractionated.

Table 2. *In vitro* antimalarial activity of crude extracts of *V. infausta* against two strains of *P. falciparum*.

	IC_{50} (μg/mL)	
Extract	D_6	W_2
Crude	24.1±1.7	25.0±0.7
n-hexane	11.8±2.9	24.5±3.1
Chloroform	14.2±1.6	21.3±4.0
n-butanol	25.6±1.8	30.3±17.5
Residual H_2O	16.8±7.8	22.4±3.3
Chloroquine	0.016	0.029
Mefloquine	0.0057	0.014
Quinine	NT	0.15

Values are the mean±SD of four separate experiments performed on different days.

Cut-off point for activity of crude extract: IC_{50} ≤49.4 μg/mL.

NT: Not tested.

The results presented here indicated that *in vitro* antimalarial testing can be carried out on crude extracts using sensitive and resistant *P. falciparum* strains. Strong antimalarial activity with IC_{50} values within the acceptable range of values for crude extracts[26] were obtained against sensitive and resistant *P. falciparum* strains.

In similar experiments, Weneen *et al*.[27] demonstrated weak antimalarial activity (IC_{50}: 49.0 μg/mL) of dichloromethane and methanol extracts of *V. infausta* root and inactivity of petroleum ether extract. Nundkumar and Ojewole[16] showed in vitro antimalarial activity of the leaf extract. It might be argued that cytotoxic natural products can give false-positive results in any *in vitro* test system.

The *in vivo* test results demonstrate the value of the extracts against *Plasmodium* species parasites. However, while *V. infausta* produces edible fruits eaten by humans and

Table 3. *In vitro* antimalarial activity of fractions of chloroform and butanol extracts of *V. infausta* against two strains of *P. falciparum*.

Chloroform extract			Butanol extract		
Fractions*	IC_{50} (μg/mL)		Fractions†	IC_{50} (μg/mL)	
	D_6	W_2		D_6	W_2
1–8	19.1±8.3	22.2±0.6	1	10.4±1.7	13.6±1.5
9–13	20.4±5.9	22.4±1.5	2	17.0±13.4	23.3±2.5
14–16	9.5±1.5	12.2±0.6	3	7.3±6.2	9.0±1.6
17–25	25.8±1.9	28.4±14.7	4	3.9±0.3	11.1±11.9
26–33	5.1±1.2	5.6±2.6	5	11.6±0.4	16.4±9.5
34–37	3.9±1.5	4.5±2.3	6	>50	>50
38–39	17.4±1.4	19.1±4.9	Chloroquine	0.016	0.029
Chloroquine	0.016	0.029	–	–	–

All values are mean±SD of four independent experiments carried out on different days.

Cut-off point for activity of crude extract: IC_{50} = 49.9 μg/mL.

*Fractions obtained from silica gel column chromatography of crude chloroform extract (Fig. 1).

†Fractions obtained from vacuum liquid chromatography of crude butanol extract (Fig. 1).

animals, extracts from other *Vangueria* species have demonstrated neither antibacterial nor antischistosomal activity.[28]

Phytochemical findings agree with those reported elsewhere[16,20,29] and suggest that its wide ethnomedicinal use could be attributed to the classes of compounds it possesses.

While the present study provides insight into the fractions from which potent antimalarial substances can be isolated, further work is planned to isolate and characterise these active compounds. □

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