IN VITRO ANTIPLASMODIAL SCREENING OF ETHNOPHARMACOLOGICALLY SELECTED SOUTH AFRICAN PLANT SPECIES USED FOR THE TREATMENT OF MALARIA

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Abstract:

Ethnopharmacological relevance: The investigated plant species are traditionally used by Venda people of South Africa, in the treatment of malaria and associated symptoms.

Aim of the study: To evaluate the *in vitro* antiplasmodial efficacy and cytotoxic properties of indigenous medicinal plants used by Venda people against malaria.

Materials and methods: *In vitro* antiplasmodial activity and cytotoxic properties were evaluated on twenty indigenous plant species. Ground plant material was extracted in dichloromethane: 50% methanol (1:1). Antiplasmodial activity was evaluated against the chloroquine-sensitive strain of *Plasmodium falciparum* (NF54). The cytotoxicity of the plant extracts were assessed against mammalian L-6 rat skeletal myoblast cells. The selectivity index (SI) values were then calculated.

Results: Of the 43 plant extracts evaluated, 10 exhibited pronounced antiplasmodial activity (IC_{50} ≤ 5µg/ml) with good therapeutic indices (SI ≥ 10). Lipophilic plant extracts were relatively more potent than polar extracts. *Tabernaemontana elegans* Stapf. (Apocynaceae) and *Vangueria infausta* Burch. subsp. *infausta* (Rubiaceae) extracts displayed significant antiplasmodial activity (IC_{50} < 2 µg/ml).

Conclusion: Findings of this study partly support the ethnomedical use of the investigated plant species by Venda people as antimalarial remedies. The study also highlights some of the knowledge gaps that require further phytochemical studies on the specified plant species.

***Keywords*:**
Antiplasmodial activity; Medicinal plants; Malaria; *Plasmodium falciparum*

GRAPHICAL ABSTRACT

Plant species	Extraction solvent	Antiplasmodial activity (*Pf*-NF54)	Cytotoxicity (L6-cells)	Selectivity Index
Albizia versicolor Welw. ex Oliv. (Fabaceae)	DCM	2.12	55.1	26
Bridelia mollis Hutch. (Phyllanthaceae)	DCM	3.06	51.4	17
Capparis tomentosa Lam. (Capparidaceae)	DCM	2.19	40.8	19
Cussonia spicata Thunb. (Araliaceae)	DCM	3.25	47.8	15
Dichrostachys cinerea Wight et Arn. (Fabaceae)	DCM	2.10	51.6	25
Rauvolfia caffra Sond. (Apocynaceae)	DCM	2.13	26.9	13
Tabernaemontana elegans Stapf. (Apocynaceae)	DCM	0.33	4.68	14
T. elegans	MeOH:H_2O	0.83	38.2	46
Vangueria infausta Burch. subsp. *infausta* (Rubiaceae)	DCM	1.84	45.7	25
Xylopia parviflora (A.Rich.) Benth. Oliv. (Annonaceae)	DCM	2.19	51.5	24
Chloroquine		0.003		
Podophyllotoxin			0.007	

1. Introduction

Despite the significant advances made in lessening the burden of malaria in recent years, the disease still remains a major public health problem affecting many people in tropical and subtropical regions (Murray et al., 2012). This is especially the case in sub-Saharan Africa where 90% of the estimated annual global malaria deaths occur (World Health Organization, 2013). Most of the conventional drugs are no longer effective due to the emergence of drug resistant strains. Additionally, some of the indispensable drugs that are still effective suffer from problems related to toxicity, prolonged treatment schedules, variable responses between strains, non-compliance by patients and inaccessibility to proper health facilities (Olasehinde et al., 2012). These factors combined with the absence of effective vaccines highlight the need for new chemotherapeutic agents with novel modes of action that may alleviate the burden of malaria. In our search for novel antimalarial plant products (Prozesky et al., 2001; Tetyana et al., 2002; Adelekan et al., 2008), twenty indigenous plant species used to treat malaria and/or malarial symptoms by Venda people, were evaluated for their antiplasmodial efficacy. We report here on the preliminary results of the study.

2. Materials and methods

2.1. Plant collection

An ethnobotanical survey and a chemotaxomic approach were followed to select and collect indigenous plant species used to treat malaria and its symptoms by Venda people. The selection of medicinal plants investigated in this study was based on informal interviews with Venda people living in Mutale Municipality of Limpopo Province. Main questions asked were; which local plants are used in cases of malaria or its related symptoms, plant parts harvested for such purposes and where are they collected. The data was gathered from Venda people and from published literature. In cases where the locally used plant species was not documented in ethnopharmacological data, the plant was not harvested. Likewise, if plants were documented in literature, and not used locally, it was not collected for this study. Plant samples from the selected twenty species (Table 1.) were collected and voucher specimens were identified and deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

Table 1. Plant species evaluated for antiplasmodial activity, their ethnomedicinal uses against malaria (Bandeira et al., 2001; Mabogo, 1990; Watt and Breyer-Brandwijk, 1962), antiplasmodial activity, cytotoxicity and selectivity indices. IC_{50} values are expressed as a mean value of two independent assays and were recorded in µg/ml.

Plant species and voucher number	**Ethnomedicinal uses**	**Plant part used** (DCM: 50%MeOH)	**$IC_{50\ (parasite)}$ (** *Pf*-NF54)	**$IC_{50\ (mammalian\ cell)}$ (**L6-cells)	**[a]Selectivity Index**
Albizia versicolor Welw. ex Oliv. (Fabaceae) Mutambapfunda, 120322	Root and stem bark are used as ingredients to prepare a polyherbal decoction taken against malaria	Roots	**2.12** / 23.8	55.1 / 42.0	**26.0** / 1.76
		Stem bark	7.08 / 27.3	72.1 / 52.3	10.18 / 1.92
Anthocleista grandiflora Gilg. (Loganiaceae) Mueneene, 120323	A decoction of the stem bark and leaves is administered in cases of malaria	Stem bark	8.69 / >50	55.6 / 70.1	6.40 / n.d.
Bridelia mollis Hutch. (Phyllanthaceae) Mukumbakumba, 120324	Root infusion from a closely related plant species, *B. micrantha,* is used against malaria-related fevers	Roots	**3.06** / 28.5	51.4 / 49.6	**16.8** / 24.6
Capparis tomentosa Lam. (Capparidaceae) Moubadali, 120325	Root decoction is drunk as an antipyretic in the treatment of malaria	Roots	**2.19** / 29.2	40.8 / 70.4	**18.6** / 2.41
Clematis brachiata Thunb. (Ranunculaceae) Tshiumbeumbe,120326	Hot root decoction is used for steaming or taken orally for malaria and colds	Roots	5.36 / >50	42.6 / 72.3	7.95 / n.d.
Clerodendrum glabrum E. Mey. (Verbenaceae) Umnukalembeba,120327	Leaf infusion is taken as a remedy for fevers associated with malaria	Leaves	8.89 / >50	62.2 / 72.7	3.02 / n.d.
Cussonia spicata Thunb. (Araliaceae) Musenzhe,120328	A root infusion made from a handful of roots are used as emetics for fevers	Root bark	**3.25** / >50	47.8 / 69.1	**14.7** / n.d.

Dichrostachys cinerea Wight et Arn. (Fabaceae) Murenzhe, 120329	Crushed roots are soaked in water and administered in cases of febrifuge	Roots	**2.10** / >50	51.6 / 65.3	**24.6** / n.d.
Diospyros mespiliformis Hochst. ex A.DC. (Ebenaceae) Musuma, 129330	Root decoction is used to alleviate febrile symptoms	Roots	4.40 / 28.4	24.3 / 60.4	5.52 / 2.13
Pappea capensis Eckl. & Zeyh. (Sapindaceae) Tshikavhavhe, 120331	Branches are boiled and taken as tea for malaria	Twigs	5.47 / 24.8	54.0 / 55.2	9.87 / 2.23
Parinari curatellifolia Planch. Ex Benth. (Rosaceae) Muvhula, 120332	Stem bark soaked together with other plant species are used for the treatment of malaria	Stem bark	6.99 / 16.9	57.6 / 55.4	8.24 / 3.28
Pyrenacantha grandiflora Baill. (Icacinaceae) Bwere, 120333	A decoction prepared from powdered roots is used for malaria	Roots	5.82 / >50	0.52 / 10.5	0.089 / n.d.
Rauvolfia caffra Sond. (Apocynaceae) Munadzi, 120334	Used as a substitute for *T. elegans* (of the same family) to treat malaria and fevers	Stem bark	**2.13** / 10.8	26.9 / 57.2	**12.6** / 5.30
Senna petersiana (Bolle) Lock. (Fabaceae) Munembenembe, 120335	Leaf infusion are taken as tea for malaria	Leaves	22.5 / 22.1	59.3 / 66.8	2.64 / 3.02
Syzygium cordatum Hochst. (Myrtaceae) Mutu, 120336	Leaf infusions administered for febrifuge and headaches related to malaria	Leaves	6.15 / 10.4	65.7 / 53.8	10.68 / 5.17
Tabernaemontana elegans Stapf.	Stem bark and root decoctions are	Stem bark	**0.331 / 0.834**	4.68 / 38.2	**14.1 / 45.8**

(Apocynaceae) Muhatu, 120337	used for febrifuge and malaria				
Vangueria infausta Burch. subsp. *infausta* (Rubiaceae) Muzwilu, 120338	Infusions made from the roots and leaves is taken orally to treat malaria	Roots	**1.84** / >50	45.7 / 71.5	**24.8** / n.d.
[b]*Ximenia americana* Linn. (Olacaceae) Muthanzwa, 120339	Root infusions are taken for febrifuge and ground root powder is applied topically for febrile headaches	Roots	28.2	69.1	2.45
Ximenia caffra Sond. (Olacaceae) Mutshili, 120340	Powdered leaves and twigs are used for fevers and febrifuge	Leaves	3.01 / >50	8.68 / >50	2.88 / n.d.
Xylopia parviflora (A.Rich.) Benth. Oliv. **(**Annonaceae) Muvhulavhusika, 120341	Hot root decoctions are used as emetics for fevers	Roots	**2.19** / 14.2	51.5 / 78.3	**23.5** / 5.51
Chloroquine		0.003			
Podophyllotoxin			0.007		

DCM: Dichloromethane, 50 % MeOH: Methanol and distilled water (1:1)
Pf-NF54: *Plasmodium falciparum* NF54 strain
L6-cells: Rat skeletal myoblast L6 cell line
[a]Selectivity index (SI): quotient of IC_{50} in L6 cells and IC_{50} against parasites
[b] *Ximenia americana* did not result in two phases between the DCM and 50% MeOH
n.d.: not determined

2.2. Extraction of plant samples

For each plant sample, 20 g of dried ground plant material was repeatedly extracted in 300 ml of dichloromethane: 50% methanol (1:1) and then filtered. The recovered filtrate was then separated. Non-polar fractions were concentrated under vacuum at 30 ºC. Methanol in the polar fractions was vaporized at 40 ºC and the resulting aqueous extracts were freeze-dried using a bench top manifold freeze dryer (Virtis). Dichloromethane and aqueous crude extracts were analysed independently.

2.3. *In vitro* antiplasmodial assay

In vitro activity of the acquired plant extracts (43) was determined following a [^{3}H]hypoxanthine incorporation assay using chloroquine sensitive (NF54) strain of *Plasmodium falciparum* as the test organism (Matile and Pink, 1990). Plant extracts were dissolved in DMSO at 10 mg/ml and added to parasite cultures incubated in RPMI 1640 medium without hypoxanthine, supplemented with HEPES (5.94 g/l), $NaHCO_3$ (2.1 g/l), neomycin (100 U/ml), AlbumaxR (5 g/l) and washed human red cells A+ at 2.5% haematocrit. Chloroquine (Sigma) was used as the standard drug. Serial drug dilutions of eleven 3-fold dilution steps were prepared. The 96-well plates were incubated in a humidified atmosphere at 37 °C, 4% CO_2, 3% O_2 and 93% N_2. After 48 h 50 µl of [^{3}H]hypoxanthine was added to each well. Plates were incubated for a further 24 h and then harvested with a Betaplate™ cell harvester (Wallac). Red blood cells were transferred onto a glass fibre filters, washed and the dried filters were then inserted into a plastic foil and counted in a Betaplate™ liquid scintillation counter (Wallac). IC_{50} values were calculated from sigmoidal inhibition curves by linear regression using Microsoft Excel (Huber and Koella, 1993).

2.4. Cytotoxicity assay

The antiproliferative activity of plant extracts was assessed on rat skeletal myoblasts L-6 cells (Ahmed et al., 1994). Assays were performed in 96-well microtiter plates, each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200mM), 10% fetal bovine serum and 4000 L-6 cells. Podophyllotoxin was used as a control. Serial drug dilutions with a range of 100 to 0.002 µg/ml were conducted. After 70 hours of incubation the plates were inspected under an inverted microscope. 10µl of Alamar was then added to each well and the plates were incubated for another 2 hours. The plates were then read with a

Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation). The IC_{50} values were calculated as in 2.2.

3. Results and discussion

The inhibitory concentration (IC_{50}) and selectivity index (SI) values of plant extracts that demonstrated significant antiplasmodial activity ($IC_{50} \leq 5$ µg/ml) when tested against the chloroquine sensitive strain of *P. falciparum* (NF54) are shown in Table 1. Selectivity index (SI) values were calculated by dividing the IC_{50} value for the cytotoxicity by the IC_{50} value of antiplasmodial activity. It is generally considered that the antimalarial efficacy of a given plant extract is not due to the *in vitro* cytotoxicity when the SI ≥ 10, therefore displaying selective antiplasmodial activity (Vonthron-Senecheau et al., 2003). For the purpose of this study, a plant extract was considered to be a potential hit for drug discovery when the IC_{50} was ≤ 5 µg/ml and an SI value of ≥ 10 could be established. Of all the 43 extracts assayed, 23% exhibited pronounced selective antiplasmodial activity.

Tabernaemontana elegans was the best candidate, as both the dichloromethane and polar extracts from its stem bark inhibited plasmodial growth at $IC_{50} = 0.33$ and $IC_{50} = 0.83$ µg/ml, respectively. With respective SI values of 14 and 46, these extracts were considered to be non-toxic to rat skeletal myoblast L6 cells. Despite the wide ethnomedical use of *T. elegans* as an antimalarial remedy (Bandeira et al., 2001), this is the first study to document its significant antiplasmodial activity. Studies conducted by Ramalhete et al., 2008 revealed moderate or no significant activity of polar leaf extracts from the same plant species. Nevertheless, studies conducted on indole alkaloids from a closely related species, *T. sessilifolia,* showed some good antiplasmodial activity (Girardot et al., 2012), which could explain the observed bioactivity.

Dichloromethane root extract of *Vangueria infausta* subsp. *infausta* showed a marked inhibitory effect ($IC_{50} = 1.84$ µg/ml, SI = 25) against *P. falciparum*. A study conducted on chloroform root bark extract from *V. infausta* subsp. *infausta* significantly inhibited two strains of *P. falciparum* at IC_{50} of 3.80 and 4.50 µg/ml (Abosi et al., 2006). Further studies are needed to determine the compounds responsible for the observed antiplasmodial activity. Chloroquine-sensitive strain of *P. falciparum* was found to be susceptible to the lipophilic extracts of *Albizia*

versicolor, *Capparis tomentosa, Dichrostachys cinerea*, *Rauvolfia caffra* and *Xylopia parviflora* at concentrations ranging from 2.10 to 2.19 µg/ml and SI values ranging between 12 and 26.

Although *Albizia* species are well documented for their strong *in vitro* as well as *in vivo* antimalarial activities (Samoylenko et al., 2009), reports on *A. versicolor* are lacking. Clarkson et al. (2004) detected a weak antiplasmodial activity (IC_{50} = 38 µg/ml) in the dichloromethane root extract *C. tomentosa*, which is relatively low compared to the results (IC_{50} = 2.19 µg/ml) found in this study. An ethanol leaf extract from *D. cinerea* showed no activity at the highest concentration (5 µg/ml) tested (Atindehou, 2004). Results from the current study do not support the relatively low antiplasmodial activity (IC_{50} ≥ 10 µg/ml) reported previously for *R. caffra* (Clarkson et al., 2004). Boyom et al. (2011) reported on the potency of methanol leaf and stem extracts of *X. parviflora* from Cameroon. In agreement with the results obtained in this study, these extracts showed high *in vitro* antiplasmodial activity (IC_{50} ≤ 5 µg/ml).

The non-polar root extracts of *Bridelia mollis* and *Cussonia spicata* demonstrated significant *in vitro* antiplasmodial activity (IC_{50} ~3 µg/ml) and selectivity for malaria parasite with SI values of 17 and 15, respectively. In South Africa, *B. mollis* is traditionally used as an antiparasitic against worms, among other uses, while a closely related species, *B. micrantha* is used against malaria-related fevers (Watt and Breyer-Brandwijk, 1962; Mabogo, 1990). Literature data on the biological activity and phytochemical constituents of *B. mollis* is limited. The genus *Cussonia* has been extensively studied for its antiplasmodial properties, and the polar bark extracts of *C. spicata* were reported to have a relatively weak activity (De Villiers et al., 2010). Results obtained in this study are consistent with those reported for other members of the same family, when extracted with non-polar solvent (Clarkson et al., 2004). Although most of the investigated plant species have been previously tested against *P*. *falciparum*, data on the compounds attributable to their respective antiplasmodial activity is very limited.

It is worth noting that antimalarial activity was mainly found in lipophilic plant extracts, which confirm earlier reports that dichloromethane extracts generally have a higher antiplasmodial activity than methanol and aqueous extracts (Irungu et al., 2007). Several species that were strongly associated with the treatment of malaria by Venda people and which are cited in ethnobotanical literature demonstrated weak

antimalarial activity in this study. Thus, traditional remedies that are inactive against the *Plasmodium* asexual erythrocytic stage may be active against the hepatocyte phase, thereby preventing infection of red blood cells (Mesia et al., 2010). Investigations into treatments for malaria should therefore be directed at targeting the various stages of *Plasmodium* life-cycle and other clinical symptoms related to the disease state (Rasoanaivo et al., 2011).

4. Conclusions

The findings of this study give a measure of credibility to the ethnomedical use of the investigated plant species by Venda people and to the rationale of an ethnopharmacological approach when bioprospecting medicinal plants for antiplasmodial lead compounds. Further phytochemical analyses are currently underway in an attempt to fractionate, isolate and identify the active constituents in extracts that demonstrated significant bioactivity.

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