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Ethanol Extracts from Selected Tanzanian Medicinal Plants Selectively Inhibit *Plasmodium falciparum* Growth *In Vitro*

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Abstract

This study aimed to assess the *in vitro* antiplasmodial and cytotoxic activities of ethanol extracts from the root barks, leaves and stem barks of seven medicinal plant species growing in Tanzania. The antiplasmodial activities of extracts against chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *Plasmodium falciparum* were determined using a SYBR green I-based fluorescence assay, while the cytotoxicity of active extracts was determined against the human HepG2 cell line using a resazurin-based assay. Out of twenty-one extracts screened, two of them, namely, DFRE from *Dracaena fragrans* root bark (IC_{50}*Pf*3D7 = 2.4 μg/mL) and EHSE from *Erythrina haerdii* stem bark (IC_{50}*Pf*3D7 = 8.6 μg/mL and IC_{50}*Pf*Dd2 = 7.6 μg/mL), exhibited good potencies against *P. falciparum* parasites. The two extracts emerged as the most promising and selective inhibitors (inhibited the growth of *P. falciparum* strains and were not toxic to the human HepG2 cell line) of chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *P. falciparum*. Eight other extracts showed moderate potencies with IC_{50} values ranging from 11–25 μg/mL against both plasmodial strains, while the remaining 11 extracts were mildly active with IC_{50} values ranging from 25.1 to 96.7 μg/mL. The results of the cytotoxicity test indicated that the median cytotoxic concentrations (CC_{50}) of the tested extracts were all ≥ 224.8 μg/mL, leading to selectivity indices (SI) > 10-fold for most of the extracts. The findings described in this article support the traditional use of the investigated medicinal plants to treat malaria.

Keywords: Antiplasmodial; Cytotoxicity; Ethanol extracts; *Plasmodium falciparum*.

Introduction

Malaria infections, caused by protozoa of the genus *Plasmodium*, are still a major public health issue, even though extensive efforts to eradicate it are in progress (Uwimana et al. 2020, Hema et al. 2021). According to a recent report published by the World Health Organization, there were estimated 241 million cases of malaria in 2020, and the estimated number of malaria deaths stood at 627000 (WHO 2022). Despite promising efforts and global programs aimed

at eradicating malaria, infections are steadily increasing due to the rapid transmission rate (Sumner et al. 2021) and the emergence of the COVID-19 pandemic in 2019 (WHO 2021). Although antimalarial chemotherapies were considered helpful, growing drug resistance to artemisinin-based combination therapies has been detected in five countries in the Greater Mekong subregion as well as in Rwanda, Uganda and Eritrea, threatening global malaria control efforts (WHO 2021). Therefore, there is an urgent need for drug alternatives endowed with novel mechanisms of action, mostly for people living in disease-endemic countries of sub-Saharan Africa. Historically, malaria chemotherapies have a strong historical link to natural products. Of note, the most successful antimalarial agents, such as artemisinin and quinine, originate from plant metabolites (Tajuddeen and Van Heerden 2019). Therefore, intensive and continuous investigations of medicinal plants could lead to the identification of novel molecules that hit alternative targets to block *P. falciparum* asexual proliferation. In fact, several plants are traditionally used in disease-endemic countries for malaria treatment or associated symptoms. However, their full potential and efficacy have been scarcely scientifically validated/documented.

Within that framework, the root of *Dracaena fragrans* is used for the treatment of malaria in East Africa as well as the treatment of hepatomegaly and rheumatism (Chhabra et al. 1984, Meksuriyen and Cordell 1988). The stem bark and root bark of *Acacia xanthophloea* are traditionally used for the treatment of malaria (Chhabra et al. 1984). Bark of *Vangueria madagascariensis* is used for the treatment of malaria, hepatitis and intestinal worms (Chhabra et al. 1984). Leaves of *Dombeya burgessiae* are used for the treatment of malaria and leprosy (Chhabra et al. 1984). The root bark of *Erythrina abyssinica*, a species closely related to *Erythrina haerdii*, is used for the treatment of malaria, gonorrhoea and abdominal pain, and as an antihelminthic (Maregesi et al. 2007). The roots of *Keetia zanzibarica* are used for the treatment of malaria, stomachaches and wounds (Chhabra et al. 1991). The roots of *Chassalia umbraticola* are used for the treatment of malaria-associated convulsions in children as well as the treatment of chronic gonorrhea and chest pains. Additionally, the leaves of *Chassalia umbraticola* are used for the control of fever (Chhabra et al. 1991). Although African traditional knowledge recognizes that these medicinal plants have useful properties against malaria, control of fever and the treatment of other diseases, scientific validation remains critical to document their efficacy against *P. falciparum* and their safety to humans. Hence, the work reported hereby provides data on the *in vitro* antiplasmodial activities and cytotoxicity of ethanol extracts of root barks, leaves and stem barks of seven selected medicinal plant species growing in Tanzania.

Materials and Methods

Collection of plant materials

The root barks, leaves and stem barks of the investigated plant species were collected in Tanzania as follows: *Acacia xanthophloea* Benth collected in May 2021 from the Mkomazi River (GPS location: 37 M 0397534 UTM 9484613; elevation 459 m) in Korogwe District; *Chassalia umbraticola* Vatke collected in May 2021 at Miomboni Street (GPS location: 37 M 0481412 UTM 9256843; elevation 102 m) in Kibaha District; *Dombeya burgessiae* Gerr. ex Harv. collected in May 2021 at the Sao-Hill plantation (GPS location 36 L 0738880 UTM 9060552; elevation 1900 m) in Mufindi District; *Dracaena fragrans* (L.) Ker Gawl. Collected in May 2021 at Mavimo Forest near Kunga Tea Estate (GPS location 37 M 434278 S 9439128 E) in Korogwe District; *Erythrina haerdii* Verdc. collected in May 2021 at Sululu Village (GPS location 341 M latitude 7.977817° S longitude 36.839937° E) in Kilombero District; *Keetia zanzibarica* (Klotzsch) Bridson collected in May 2021 at Miomboni Street (GPS location 37 M 0481412 UTM 9256843) in Kibaha District; *Vangueria madagascariensis* J. F. Gmel. collected in May 2021 at Miomboni Street (GPS location 37 M 0481408 UTM 9256847; elevation 107 m) in Kibaha District. The

plant species were identified in the field during their collection and confirmed in the Herbarium of Botany Department, University of Dar es Salaam, where voucher specimens FMM 4002, FMM 3997, FMM 4004, FMM 4001, FMM 4003, FMM 3998 and FMM 3999 for *A. xanthophloea*, *C. umbraticola*, *D. burgessiae*, *D. fragrans*, *E. haerdii*, *K. zanzibarica* and *V. madagascariensis*, respectively, are preserved.

Extraction of plant materials

Twenty grams (20 g) of air-dried and pulverized root bark, leaves and stem bark of selected plants were each extracted by maceration using ethanol at room temperature for 48 hours. The concentration of the extracts to remove the solvent was performed under reduced pressure using a rotary evaporator to obtain crude extracts for subsequent biological assays.

Determination of the *in vitro* antiplasmodial activity

The *in vitro* antiplasmodial assays of ethanol extracts were carried out at the Antimicrobial and Biocontrol Agents Unit, University of Yaounde 1, Cameroon, using chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *Plasmodium falciparum*.

In vitro* cultivation of *Plasmodium falciparum

The plasmodial strains used were maintained in complete Roswell Park Memorial Institute (RPMI 1640) culture medium in fresh O^+ human erythrocytes suspended at 4% hematocrit in 16.2 g/L RPMI 1640 containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 11.11 mM glucose, 0.2% sodium bicarbonate, 0.5% Albumax I (Gibco, USA), 0.1 mM hypoxanthine, 0.016 mM thymidine (Gibco, USA), and 50 mg/mL gentamicin (Gibco, China) and incubated at 37 °C under a humidified atmosphere containing 5% CO_2. The medium was replaced with fresh complete medium daily to propagate the culture. Giemsa-stained thin blood smears were examined microscopically under oil immersion to monitor cell cycle transition and parasitaemia (Trager and Jensen 1976).

Synchronization of *Plasmodium falciparum* parasite culture

Prior to antiplasmodial activity testing, synchronized ring stage parasites were obtained by 5% (w/v) sorbitol treatment for 10 min as previously described (Lambros and Vanderberg 1979). The homogeneity of the synchronized ring-stage parasite culture was verified microscopically using Giemsa-stained blood smears under oil immersion before the antiplasmodial assay.

***In vitro* antiplasmodial screening of ethanol extracts**

Test samples were assessed for antiplasmodial activities against both 3D7 and Dd2 strains of *P. falciparum* using the SYBR Green I-based fluorescence assay as described by Smilkstein et al. (2004). Briefly, 100 µL cultures of sorbitol–synchronized ring stage parasites were incubated under normal culture conditions as described above at 1% parasitemia and 2% hematocrit in flat-bottomed 96-well plates in the absence or presence of 5-fold serially diluted concentrations of test samples (0.064 to 1000 µg/mL) for 48 h. Chloroquine and artemisinin were used as positive controls (0% growth) and tested at 1 µM, while 0.4% *v/v* DMSO was used as the negative control (100% growth). Following incubation, 100 µL of SYBR Green I lysis buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (*w/v*) saponin, and 0.08% (*v/v*) Triton X-100] was added to each well, mixed gently twice and incubated in the dark at 37 °C for 1 hour. Fluorescence was measured with a Magelan Infinite M200 fluorescence microplate reader (Tecan) with excitation and emission wavelength bands centered at 485 and 538 nm, respectively. Mean fluorescence counts were normalized to percent control activity using Microsoft Excel software, and the 50% inhibitory concentrations (IC_{50}) were calculated using Prism 8.0 software (GraphPad) with data fitted by nonlinear

regression to the following variable slope sigmoidal concentration–response formula: $y = 100/[1 + 10^{(\log IC50/99-x)H}]$, where H is the Hill coefficient or slope factor (Singh and Rosenthal 2001).

Determination of the cytotoxicity of ethanol extracts against HepG2 cells

Cytotoxicity tests of ethanol extracts were carried out at the Antimicrobial and Biocontrol Agents Unit, University of Yaounde 1, Cameroon.

Maintenance of mammalian cells

The human HepG2 cell line (ATCC HB-8065) was grown in T-25 vented cap culture flasks using complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% (v/v) penicillin–streptomycin and incubated at 37 °C in an atmosphere containing 5% CO_2. The medium was renewed every 72 hours, and cell growth was assessed using an inverted microscope (Lumascope LS520). Subculture was performed when the cells reached ~80–90% confluence by detachment with 0.25% trypsin-EDTA followed by centrifugation at 1800 rpm for 5 min. The resulting pellet was resuspended and counted in a Neubauer chamber in the presence of trypan blue to exclude nonviable blue-coloured cells. Once the cell load was estimated, they were either used for the next passage in a new flask or processed for the cytotoxicity assay.

***In vitro* resazurin-based cytotoxicity assay**

The cytotoxicity of promising extracts was assessed in a 96-well tissue culture-treated plate as previously described by Bowling et al. (2012). Briefly, HepG2 cells at a density of 10^4 cells per well were plated in 100 µL of complete Dulbecco's modified Eagle's medium (DMEM) and incubated overnight to allow cell attachment. Plates were controlled under an inverted fluorescence microscope (Lumascope LS520) to assure adherence, sterility and cell integrity. Thereafter, the culture medium from each well was carefully aspirated and replaced with 90 µL of fresh complete medium followed by the addition of 10 µL of serial 5-fold dilutions of extract solutions. Podophyllotoxin (100–0.16 µM) and 0.5% DMSO (100% cell viability) were also included in assay plates as positive and negative controls, respectively. After an incubation period of 48 h at 37 °C in a humidified atmosphere with 5% CO_2, 10 µL of a stock solution of resazurin (0.15 mg/mL in PBS) was added to each well and incubated for an additional 4 h. Fluorescence was then read using a Magelan Infinite M200 fluorescence microplate reader (Tecan) with excitation and emission wavelengths of 530 and 590 nm, respectively. The percentage of cell viability was calculated from readouts, and the median cytotoxic concentration (CC_{50}) for each extract was deduced from concentration–response curves using GraphPad Prism 8.0 software as described above. Selectivity indices were then determined for each test substance as follows: Selectivity Index = $CC_{50(\text{HepG2})}/IC_{50(\textit{P. falciparum})}$.

Results and Discussion

The *in vitro* antiplasmodial activities of ethanol extracts of root barks, leaves and stem barks of the selected plant species were determined against chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *P. falciparum,* and the results (Table 1) were expressed as 50% inhibitory concentrations (IC_{50}). Artemisinin exhibited IC_{50} values of 0.03 µM against the 3D7 strain and 0.04 µM against the Dd2 strain, while chloroquine showed IC_{50} values of 0.02 µM against the 3D7 strain and 0.64 µM against the Dd2 strain. Out of the twenty-one ethanol extracts screened, two, namely, DFRE from *Dracaena fragrans* root bark and EHSE from *Erythrina haerdii* stem bark, exhibited good activities against the chloroquine-sensitive (*Pf*3D7) strain of *P. falciparum* with IC_{50} values of 2.4 and 8.6 µg/mL, respectively. The remaining extracts elicited moderate to mild activities with IC_{50} values ranging from 11.0 to 96.7 µg/mL. On the other hand, while screening the 21 extracts against the multidrug-resistant (*Pf*Dd2) strain of *P. falciparum*, only EHSE from *Erythrina haerdii* stem bark (IC_{50}*Pf*Dd2 = 7.6 µg/mL)

exhibited good activity, while the other extracts showed moderate to mild activities with IC_{50} values ranging from 10.8 to 79.5 μg/mL.

To determine the selectivity profile of the antiplasmodial extracts, their cytotoxicity was evaluated against the normal human HepG2 cell line (ATCC HB-8065). The results shown in Table 1 indicated that the median cytotoxic concentrations (CC_{50}) of the tested extracts were all ≥ 224.8 μg/mL, leading to selectivity indices (SI) > 10-fold for most of the extracts. Thus, the investigated extracts selectively targeted *P. falciparum* rather than normal human HepG2 cells. Of note, the most potent antiplasmodial extracts (DFRE from *Dracaena fragrans* root bark and EHSE from *Erythrina haerdii* stem bark) were the most selective, with selectivity indices greater than 60-fold toward the chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) parasitic strains.

The antiplasmodial potency and selectivity of the DFRE extract (IC_{50}*Pf*3D7 = 2.4 μg/mL/SI= 296.6 and IC_{50}*Pf*Dd2 = 10.8 μg/mL/SI = 66.8) further support the traditional use of *D. fragrans* roots for the treatment of malaria in East Africa (Chhabra et al. 1984, Meksuriyen and Cordell 1988). The second most active extract was EHSE from *Erythrina haerdii* stem bark (IC_{50}*Pf*3D7 = 8.6 μg/mL/SI = 61.8 and IC_{50}*Pf*Dd2 = 7.6 μg/mL/SI = 70.3). In addition, the *Erythrina haerdii* root bark extract (EHRE) displayed the third highest potency against the sensitive (3D7) and multidrug-resistant (Dd2) plasmodial strains (IC_{50}*Pf*3D7 = 11.0 μg/mL and IC_{50}*Pf*Dd2 = 15.2 μg/mL) and selectivity indices greater than 29 for both parasite strains (Table 1). The displayed antiplasmodial potency and selectivity of extracts from *Erythrina haerdii* suggest that they likely contain bioactive metabolites (flavonoids and isoflavonoids) similar to those present in *E. abyssinica*, a closely related species that is traditionally used for the treatment of malaria, gonorrhoea, and abdominal pain and as an antihelminthic (Yenesew et al. 2004, Maregesi et al. 2007). The other extracts displayed moderate to mild antiplasmodial activities. In this regard, the *Dracaena fragrans* stem bark ethanol extract (DFSE) exhibited activity against the *Pf*3D7 and *Pf*Dd2 strains with IC_{50} values of 12.8 μg/mL and 19.2 μg/mL and selectivity indices of 43 and 28.6, respectively (Table 1). This is an indication that similar bioactive metabolites that are found in the root bark extract (DFRE) that elicited the highest potency against *P. falciparum* are likely present in the stem bark extract (DFSE) of *D. fragrans*.

The ethanol extract of the stem bark of *Acacia xanthophloea* (AXSE) exhibited antiplasmodial activity against the chloroquine-sensitive (*Pf*3D7) and multidrug-resistant (*Pf*Dd2) plasmodial strains of *P. falciparum* with IC_{50} values of 13.3 μg/mL and 16.3 μg/mL, respectively (Table 1). The potency of this extract supports the claims that *A. xanthophloea* stem bark is used for the treatment of malaria (Chhabra et al. 1984). The SI of this extract toward the *Pf*3D7 and *Pf*Dd2 strains was 30 and 24.6, respectively. *Vangueria madagascariensis* leaf ethanol extract (VMLE) also exhibited moderate antiplasmodial activity against the *Pf*3D7 and *Pf*Dd2 strains with IC_{50} values of 13.5 μg/mL and 20.8 μg/mL and SI values of 35.5 and 23.1, respectively.

Overall, the findings reported in this paper support the ethnobotanical uses of some of the investigated plant species in the treatment of malaria. The cytotoxicity profiles of the studied extracts against normal mammalian cells provide an extent of validation for their use by humans for therapeutic purposes. However, *in vivo* experimental data on antimalarial efficacy and toxicology are required to fully validate the use of promising extracts for the treatment of malaria in indigenous practices.

Table 1: Antiplasmodial and cytotoxic activities of ethanol extracts from root bark, leaves and stem bark of selected medicinal plant species

Part of plant species investigated	Extract code	IC_{50} ± SD(µg/mL)		Selectivity against HepG2 cells		
		*Pf*3D7	*Pf*Dd2	Mean CC_{50} (µg/mL)	SI (*Pf*3D7)	SI (*Pf*Dd2)
Acacia xanthophloea Benth. leaves (Leguminosae)	AXLE	23.3 ± 0.5	27.4 ± 1.9	408.0	17.5	14.9
Acacia xanthophloea root bark	AXRE	21.5 ± 0.1	23.1 ± 1.5	488.2	22.7	21.2
Acacia xanthophloea stem bark	AXSE	13.3 ± 3.2	16.3 ± 0.8	400.5	30.0	24.6
Chassalia umbraticola Vatke leaves (Rubiaceae)	CULE	39.3 ± 3.5	79.5 ± 18.7	837.2	21.3	10.5
Chassalia umbraticola root bark	CURE	55.3 ± 19.3	26.9 ± 9.0	>1000	>18	>37
Chassalia umbraticola stem bark	CUSE	24.3 ± 3.6	37.5 ± 0.5	493.1	20.3	13.1
Dombeya burgessiae Gerr. ex Harv. leaves (Malvaceae)	DBLE	15.7 ± 2.0	17.6 ± 0.9	483.9	30.8	27.6
Dombeya burgessiae root bark	DBRE	24.0 ± 1.7	24.8 ± 3.8	264.7	11.0	10.7
Dombeya burgessiae stem bark	DBSE	21.9 ± 0.8	27.7 ± 7.1	552.8	25.3	19.9
Dracaena fragrans (L.) Ker Gawl. leaves (Asparagaceae)	DFLE	46.3 ± 0.4	35.7 ± 7.6	496.6	10.7	13.9
Dracaena fragrans root bark	DFRE	2.4 ± 0.2	10.8 ± 3.0	721.0	296.6	66.8
Dracaena fragrans stem bark	DFSE	12.8 ± 0.04	19.2 ± 5.5	548.5	43.0	28.6
Erythrina haerdii Verdc. leaves (Leguminosae)	EHLE	25.6 ± 2.5	36.4 ± 1.9	384.6	15.0	10.6
Erythrina haerdii root bark	EHRE	11.0 ± 0.7	15.2 ± 0.9	454.3	41.1	29.9
Erythrina haerdii stem bark	EHSE	8.6 ± 0.8	7.6 ± 0.7	530.6	61.8	70.3
Keetia zanzibarica (Klotzsch) Bridson leaves (Rubiaceae)	KZLE	20.1 ± 0.3	14.8 ± 3.5	764.4	38.1	51.5
Keetia zanzibarica root bark	KZRE	96.7 ± 5.7	>100	>1000	>10	ND
Keetia zanzibarica stem bark	KZSE	53.8 ± 8.5	43.0 ± 6.1	>1000	>18	>23
Vangueria madagascariensis J. F. Gmel. leaves (Rubiaceae)	VMLE	13.5 ± 0.7	20.8 ± 1.9	479.1	35.5	23.1
Vangueria madagascariensis root bark	VMRE	31.1 ± 2.2	24.4 ± 0.6	765.1	24.6	31.4
Vangueria madagascariensis stem bark	VMSE	26.2 ± 3.5	18.1 ± 1.8	224.8	8.6	12.5
Artemisinin (µM) (positive control)		0.03 ± 0.00	0.04 ± 0.01			
Chloroquine (µM) (positive control)		0.02 ± 0.00	0.64 ± 0.08			

IC_{50} = median inhibitory concentration; CC_{50} = median cytotoxic concentration; SI = selectivity index (CC_{50}HepG2/IC_{50}*P. falciparum*); SD = standard deviation. Extract code abbreviation note: first letter (generic name), second letter (specific name), third letter (part of plant collected, L = leaf, R = root bark, S = stem bark), the last letter "E" = ethanol (solvent used for extraction).

Conclusion

This study has demonstrated the antiplasmodial potential and safety of extracts from selected Tanzanian medicinal plants. The findings support the traditional uses of the investigated medicinal plants to treat malaria in eastern Africa. Ethanol extracts of *Dracaena fragrans* root bark (DFRE) and *Erythrina haerdii* stem bark (EHSE) have emerged as the most promising and selective

inhibitors of chloroquine-sensitive (3D7) and multidrug-resistant (Dd2) strains of *P. falciparum.* Further bioactivity-guided isolation is required to identify antiplasmodial hit compounds from promising extracts to support further antimalarial drug discovery efforts.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest associated with this work.

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