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Research Article

In Vitro Antiplasmodial Activity and Cytotoxicity of Extracts of Selected Medicinal Plants Used by Traditional Healers of Western Cameroon

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Medicinal plants play a key role in malaria control in Africa, especially in remote areas where health facilities are limited. In order to assess their acclaimed potentials, eleven extracts were prepared from seven selected plants commonly used in Western Cameroon, and tested both for their antiplasmodial activity and cytotoxicity. The antiplasmodial activity was assessed using Lactate Dehydrogenase Assay (pLDH) and the cytotoxicity estimated on LLC-MK2 monkey kidney epithelial cells. Seven extracts from five different plants were significantly active, with very weak or no cytotoxicity. The *Dacryodes edulis* leaves showed the highest activity (IC_{50} of 6.45 μg/mL on 3D7 and 8.2 μg/mL on DD2) followed by the leaves of *Vernonia amygdalina* (IC_{50} of 8.72 and 11.27 μg/mL on 3D7 and DD2 resp.) and roots of *V. amygdalina* (IC_{50} of 8.72 μg/mL on 3D7), *Coula edulis* leaves (IC_{50} of 13.80 μg/mL and 5.79 μg/mL on 3D7 and DD2 resp.), *Eucalyptus globulus* leaves (IC_{50} of 16.80 μg/mL and 26.45 μg/mL on 3D7 and DD2) and *Cuviera longiflora* stem bark (IC_{50} of 20.24 μg/mL and 13.91 μg/mL on 3D7 and DD2). These findings justify the use of five of the seven plants in malaria treatment by traditional healers of Western Cameroon.

1. Introduction

Malaria is the world's most important parasitic disease especially when *Plasmodium falciparum* is the causative agent. From the recent World Malaria Report, there were an estimated 225 million malaria cases, with 800,000 deaths among the 3 billion people at risk in 2009. About 91% of total deaths occurred in Africa with pregnant women and children under 5 years being the most affected groups of the populations [1]. Cameroon figures among the 18 countries bearing 90% of malaria deaths in Africa, with 71% of its population living in high-transmission areas [2]. In the western region of Cameroon, for example, malaria was shown to be the most important cause of infant mortality causing about 45% deaths and 54% hospitalizations for children under five [3]. In the absence of an effective vaccine, the fight against malaria relies mainly on chemotherapy and vector control. The resistance of *Plasmodium falciparum* to the commonly used antimalarial drugs including the newly introduced Artemisinins has resulted in resurgence in treatment failures [4, 5]. Hence, new highly efficacious and affordable antimalarial agents are urgently needed. For hundreds of years, plants have constituted the basis of traditional medicine systems and natural products have been a good source for drug development. Some examples are Quinine and Artemisinin that have been used successfully against resistant strains of malaria parasites. In some rural areas of Cameroon, antimalarial traditional medicines are even preferred to pharmaceutical compound drugs, suggesting that the herbal preparations used by traditional healers contain useful active ingredients [6]. The office of Traditional Medicine at the Public Health Ministry promotes and coordinates Traditional Medicine Practice in Cameroon. However many gaps still remain to fully exploit the potential of herbal medicines as a solution to the malaria burden and this demands combined efforts from public sector, research institutions, funding bodies and pharmaceutical groups, traditional healers, and the populations themselves.

A previous review by Titanji et al. [5] showed that over 200 different plant species have been identified in Cameroon for their use as antimalarial in folk medicine. But only 26 species had undergone full investigation leading to isolation and testing of pure products, up to 137 species remaining untested [7]. Therefore, there is a need to pursue investigations on the plants so far identified in order to foster and standardize their use either as herbal medicines or sources of new drug candidate molecules. The present study thus aims at screening the crude extracts from seven widely used plants, namely, methylene chloride/methanol (1 : 1) extracts of *Cuviera longiflora, Dacryodes edulis* and *Eucalyptus globules* leaves, *Vernonia amygdalina* leaves and roots, the hexane and methylene chloride extracts of *Kotschya speciosa* aerial part and the methylene chloride extracts of the whole plant, the methanol extract of *Coula edulis* stem bark, the methylene chloride/methanol (1 : 1), and methylene chloride extracts of *Vismia guinensis* stem bark, for their postulated antiplasmodial activity.

2. Materials and Methods

2.1. Collection of Plants Materials. All the plant parts were collected in Batcham (Bamboutos division, Western Region Cameroon) in April 2008. The sample identification was confirmed at the Cameroon National Herbarium by matching them with existing Voucher specimens deposited at the same institution in Yaounde.

2.2. Preparation of Crude Extracts. The air dried and powdered plant material (3.5 Kg of each) was macerated for three days at room temperature separately and concurrently in 10 L of each of the following solvents: a methylene chloride/methanol (1 : 1) mixture, methylene chloride, hexane and methanol. The mixture was filtered with Whatman paper, then concentrated to dryness to viscous residues using a Rotavapor system (BÜCHI Labortechnik AG, Switzerland) [5]. The crude extracts prepared were stored at 4°C for further use.

2.3. Parasite Strains. The 3D7 (MRA-102) strain was obtained from MR4 (Manassas, VA, USA) and maintained in continuous culture with back up stored in liquid nitrogen.

The DD2 strain was kindly donated to our research team by Prof. Alex Rowe from C.I.I.E of Edinburgh University, UK.

2.4. Plasmodium Falciparum Culture and Maintenance. *P. falciparum* were grown and maintained in culture using the method of Trager and Jensen with some modification [8]. All the chemicals except Albumax II (Gibco; Invitrogen, USA) were ordered from Sigma-Aldrich Inc (Germany). Cultures consisted of a 4% hematocrit suspension of O+ human erythrocytes in RPMI 1640 medium supplemented with Gentamicin solution at 0.01 mg/mL, 25 mM HEPES buffer, 25 mM $NaHCO_3$, and 1% Albumax II. Cultures were fed with a gas mixture containing 5% CO_2 and incubated at 37°C. The estimation of the parasitaemia as well as parasite visualisation before incubation was done using both Fluorescence (Acridine Orange) and normal light (Giemsa stain) microscopes.

2.5. Determination of In Vitro Antiplasmodial Activity. Drug sensitivity assay was carried out in 96-well microtitration plates as described by Desjardins et al. [9] with some modifications [10]. The crude extracts were dissolved in dimethyl sulfoxide (200 μL) and prediluted with culture medium to make a final concentration of 1000 μg/mL. All stock solutions were sterilized by passing them through a 0.2 μm syringe filter and stored at −20°C until required. Similarly chloroquine stock (2 μg/mL) used as positive control, was prepared from liquid Chloroquine phosphate (Greenfield Pharmaceutical LTD, Jiang Su, China).

Dose-response assay was carried out to obtain the 50% inhibitory concentration (IC_{50}) of the individual drugs. Ring stage infected erythrocytes (100 μL per well with 2% hematocrit and 1% parasitaemia) were incubated in triplicate with twofold serial dilution of each drug for 48 hours. Each experiment was performed in duplicate separate experiment. Parasitaemia was measured using the parasite lactate dehydrogenase (pLDH) assay [8].

2.6. Lactate Dehydrogenase (pLDH) Assay. While incubation of parasites with products was going on, the two reagents for detecting and measuring the LDH enzyme were prepared [8]. The first of these is the Malstat reagent, which was made by dissolving 400 μL of Triton X-100 in 80 mL of deionised water, adding L-lactate (4.00 g), Tris buffer (1.32 g), and 0.022 g of 3-acetyl pyridine adenine dinucleotide (APAD), adjusting the pH to 9 with hydrochloric acid, and bringing the volume up to 200 mL with deionised water. The second reagent is NBT/PES solution, and it was prepared by dissolving nitro blue tetrazolium salt (0.160 g) and phenazine ethosulfate (0.008 g) in 100 mL of deionized water. The solution was stored in a foil-covered container and kept in the refrigerator until required. All reagents for preparing the Malstat reagent and NBT/PES solution were purchased from Sigma-Aldrich Inc. When incubation was complete, plates were harvested and subjected to three 20-minute freeze–thaw cycles to release the cell content and resuspend the culture. Thereafter, 100 μL of Malstat reagent and 25 μL of NBT/PES solution were added to each well of a new flat-bottomed 96-well microtiter plate in triplicate. Thereafter, the culture in each well was mixed and 20 μL of the culture taken from each well and added to the corresponding well of the Malstat plate, 25 μL of NTB/PES were then added to each well, thereby initiating the lactate dehydrogenase reaction. Colour development of the LDH plate was monitored colorimetrically at 650 nm with the aid of a plate reader (Emax-Molecular Devices Corporation, California, USA) after an hour of incubation in the dark.

2.7. Analysis of Test Results from the LDH Assay. The LDH assay generates optical density (OD) values at various concentrations of the drug as raw data. OD values from control wells represent the maximum amount of LDH that is produced by parasites and OD values from blank

wells represent background LDH activity. A 100% growth value, which corresponds to maximum LDH activity, was obtained by subtracting the mean OD value of blank wells from that of control wells. Likewise, the growth value at each concentration of the drug was obtained by adjusting OD values from drug-treated wells for background LDH activity (parasite-free red blood cells). These values were then expressed as a percentage of the 100% growth value and plotted against corresponding concentrations of the drug using GraphPad Software (San Diego California USA, http://www.graphpad.com/) to generate log dose-response curves from which IC_{50} values were obtained. Each extract were tested in triplicate. IC_{50} values were log transformed and expressed as geometric mean IC_{50} and 95% confidence intervals for the geometric mean calculated for the replicates. The OD values obtained were also used to verify the different IC_{50s} and determine IC_{95} values using the software HN-NonLin V1.1 (http://www.malaria.farch.net/). The different IC_{50s} values obtained were then grouped and their means calculated and compared among themselves using Independent-Samples *t*-test.

2.8. Cytotoxicity Study of Extracts. The cytotoxicity of the extracts and pure compounds was estimated against LLC-MK2 (ATCC, USA) monkey kidney epithelial cells according to the procedure previously described [11] with some modifications. Cells were cultured in the same conditions as *P. falciparum*. For the determination of the toxicity of the extracts, cells were distributed in 96-well plates at 20,000 cells per well in 100 μL culture medium. Cells were allowed to attach for 24 hours. The medium was removed completely the following day, and 100 μL of fresh medium added to all the wells. Then, 100 μL of crude extract (1000 μg/mL) were added in triplicate in row H and twofold serial dilution made upward ending at row B, and 100 μL of medium added to all the wells to have concentration range of 250–3.90, cells at raw A serving as control without drug. The plates were incubated for 72 hours at 37°C in 5% CO_2 air. Cells concentration and viability in the presence of extract or pure compounds were compared with that of control cultures without extracts. The definition of the cytotoxicity used [11] was CC_{50} < 1.0 μg/ml (high cytotoxicity), CC_{50} 1.0–10.0 μg/mL (moderate), CC_{50} 10.0–30.0 μg/mL (mild), and CC_{50} > 30 μg/mL (nontoxic). The selectivity index defined as $SI = CC_{50}/IC_{50}$ was also calculated.

3. Results

Table 1 summarizes some characteristics of the plant extracts tested in the present study. It can be seen that *E. globulus* leaves had the highest extraction yield, followed by *V. amygdalina* leaves, whereas *K. speciosa* in hexane recorded the lowest yield.

Table 2 presents the results of antiplasmodial activity and cytotoxicity of the ten extracts tested together with the activity of Chloroquine phosphate. The values of IC_{50} range from 8.72 to 64.30 μg/mL. Seven of the eleven extracts exhibited significant antiplasmodial activity (IC_{50} < 50 μg/mL) on both 3D7 and DD2 strains of malaria parasite. These active extracts were prepared from five different plant species: *D. edulis*, *C. longiflora, E. globulus*, *K. speciosa*, and *V. amygdalina* and *C. edulis*. The most active extracts were from *D. edulis* followed by *V. amygdalina* with IC_{50} values below 10 μg/mL on 3D7 and the lowest IC_{50} values on the Chloroquine-resistant strain. No sign of cytotoxicity was observed with extracts from *D. edulis*, *V. guinensis*, *V. amygdalina*, and methylene chloride extracts of *K. speciosa*, whereas extracts from *C. edulis*, *E. globulus*, and *C. longiflora* showed a very low cytotoxicity on LLC/MK2 epithelial cells.

4. Discussion

Eleven crude extracts prepared from seven different plant species from Western Cameroon were screened *in vitro* for their antiplasmodial activity and cytotoxicity.

E. globulus recorded the highest extraction yield (2.02%), followed by *V. amygdalina* root (1.85%), whereas aerial part of *Kotschya speciosa* (KSH) had the lowest yield. In general, extraction yields were the highest with Methylene Chloride/Methanol (1 : 1) mixture. This may be because the solvent system extracts both polar and nonpolar constituents, unlike the ones used in extracting the other plants which were either polar or nonpolar solvents.

The antiplasmodial activity of *Vernonia amygdalina* and *Eucalyptus globulus* was previously reported [5]. IC_{50} values range from 6.45 to 65.29 μg/mL, with three extracts (DEL, VAL and VAR) exhibiting high activity (1< IC_{50} ≤ 10 μg/mL), four with moderate activity on both chloroquine sensible and multiresistant strains of *P. falciparum* (10 < IC_{50} ≤ 25 μg/mL), and one (VGE) with weak activity (25 < IC_{50} ≤ 50 μg/mL), whereas three of them (KS, KSR and VGS) showed very weak antiplasmodial activity (50 < IC_{50} ≤ 100 μg/mL).

D. edulis exhibited the highest activity, followed by *V. amygdalina, Coula edulis*, and *Eucalyptus globulus*. *D. edulis* has a long history of use in folk medicine. It is a multipurpose plant in African folk medicine, as its various parts are used to treat several diseases. It is used in traditional medicine as a remedy for parasitic skin diseases, jigger, mouthwash, tonsillitis, sickle cell, and malaria [12, 13]. Phytochemical analysis of the leaves revealed the presence of phenolic compounds [14], and several compounds from this class have previously been shown as having antiplasmodial activity [15]. Their presence may, therefore, explain the antiplasmodial activity observed. In Western Cameroon, the leaves of *D. edulis* are boiled with those of *Cymbopogon citratus* and *Mangifera indica* in water to form a concoction against malaria, but information concerning *in vitro* antiplasmodial of the leaves extracts has not been reported earlier. Our findings justifying the use of *D. edulis* as antimalarial could, therefore, be used as background knowledge for further studies of the plant as potential source for antimalarial leads.

The *V. amygdalina* leaves are commonly used to treat malaria and other ailments [4]. Phytochemical screening of *V. amygdalina* revealed the presence of alkaloids, glycosides, saponins, tanins, flavonoids, proteins, and carbohydrates

Table 1: Plant parts used and their extraction yields.

No.	Scientific name (Family)	Local name (Locality)	Part used	Solvent	Extraction yield (% w/w)
1	*Cuviera longiflora* (Rubiaceae)	ND	Leaves	CH_2Cl_2/MeOH (1 : 1)	1.40
2	*Dacryodes edulis* (Burseraceae)	Zo'o (Batcham)	Leaves	CH_2Cl_2/MeOH (1 : 1)	1.68
3	*Eucalyptus globules* (Myrtaceae)	Klatusse (Batcham)	Leaves	CH_2Cl_2/MeOH (1 : 1)	2.02
4	*Kotschya speciosa* (Leguminoceae)	ND	Whole plan	CH_2Cl_2	0.86
			Aerial part	Hexane	0.52
			Aerial part	CH_2Cl_2	0.64
5	*Coula edulis* (Olacaceae)	Walnut (Batcham)	Stem bark	MeOH	0.78
6	*Vernonia amygdalina* (Asteraceae)	Bitter leaf (Batcham)	Leaves	CH_2Cl_2	1.75
			Root bark	CH_2Cl_2	1.85
7	*Vismia guinensis* (Asteraceae)	ND	Stem bark	CH_2Cl_2	1.12
				CH_2Cl_2/MeOH (1 : 1)	1.84

CH_2Cl_2/MeOH (1 : 1): methylene chloride/methanol mixture (1 : 1); CH_2Cl_2: methylene chloride; MeOH: methanol; %w/w: percentage weight/Weight; ND: not determined.

Table 2: Antiplasmodial activity and cytotoxicity of the tested products.

Scientific name (Family)	Part used (Code)	IC_{50} on 3D7 (μg/mL)	IC_{50} on DD2 (μg/mL)	CC_{50} on LLC/MK2 (μg/mL)	SI on 3D7 (SI on DD2)
Cuviera longiflora	Leaves (CLL)	20.24 ± 4.95	13.91 ± 0.65	250	12.35 (17.91)
Dacryodes edulis (Burseraceae)	Leaves (DEL)	6.45 ± 0.88	8.62 ± 1.44	>1000	ND
Eucalyptus globulus	Leaves (EGL)	16.80 ± 8.35	26.45 ± 3.32	208.33	12.40 (7.88)
Kotschya speciosa	Whole plant (KS)	48.29 ± 4.46	108.89 ± 17.21	>1000	ND
	Aerial part (KSH)	22.61 ± 10.34	ND	625	27.64
	Aerial part (KSR)	64.30 ± 12.76	59.30 ± 8.32	>1000	ND
Coula edulis	Stem bark (CE)	13.80 ± 3.25	5.79 ± 1.33	178.57	12.94 (30.84)
Vernonia amygdalina	Leaves (VAL)	8.72 ± 2.11	11.27 ± 2.06	>1000	ND
	Root bark (VAR)	9.01 ± 1.11	ND	>1000	ND
Vismia guinensis	Stem bark (VGE)	43.42 ± 2.76	4.735 ± 3.54	>1000	ND
	Stem bark (VGS)	65.29 ± 7.58	62.5 ± 12.45	>1000	ND
Chloroquine		0.10	0.95 ± 0.01	—	—

IC_{50}: inhibition 50%, CC_{50}: cytotoxic concentration 50%, each value represents the average and standard deviation obtained from 6 different measurements; ND: not determined.

[16]. Alkaloids are one of the most important classes of natural products providing drugs since ancient times. The outstanding example is quinine from *Cinchona succirubra* (Rubiaceae) used for the treatment of malaria for more than three centuries [17]. Equally, flavonoids are shown to inhibit the malaria parasite growth through the L-glutamine pathway although their exact mechanism of action is yet to be clarified [18]. Several plants of the Asteraceae (Compositeae) family have been revealed as good source for antimalarials, the most famous one being *Artemisia annua* the Chinese herb from which artemisinin (quinghaosu) was isolated [19]. Equally, Lupeol was identified as a compound responsible for the antimalarial activity of the *Vernonia brasiliana* leaves, inhibiting the *P. falciparum* growth by 45% when tested at 25 μ/mL. The high activity observed from the present investigation with both leaves and roots of *V. amygdalina* which is a member of this family thus supports the use of this plant for malaria by traditional healers.

Phytochemical screening of *Coula edulis* by Adebayo-Tayo and Ajibesin [20] revealed the presence of alkaloids, glycosides, saponins, tanins, flavonoids, and terpenes [20]. *C. edulis* has been shown to exhibit a wide range of antimicrobial and antifungal activities but from the literature, the antiplasmodial activity of this species has not previously been reported. The presence of compounds like alkaloids, saponins, flavonoids, and glycosides may be the source of the

antiplasmodial activity exhibited by this plant. Our findings, therefore, corroborate the use of *C. edulis* as antimalarial in Cameroonian folk medicine.

Eucalyptus globulus which is widely employed by traditional healers of Western Cameroon for malaria has previously been studied for its postulated properties [4]. Previous studies have revealed *E. globulus* to be a good source of antimicrobial leads [21]. The leaves of *Eucalyptus robusta* (another plant of the same genus) are used in China for the treatment of dysentery, malaria, and bacterial diseases. Three active compounds, Robustaol A, Robustadial A, and Robustadial B, were isolated from the ethanol extract of the leaves [22]. The present work on the crude extract from leaves agree with these previous findings, justifying the wide use of *E. globulus* as antimalarial by endogenous traditional healers of Western Cameroon.

Cuviera longiflora is a medicinal plant commonly used across the continent and out of Africa to treat various ailments. No report on investigation of this plant for its biological activity was found. The results obtained from our study reveal that *C. longiflora* has antimalarial properties. Further phytochemical screening may lead to identification of ingredients responsible for the observed antiplasmodial activity of the plant.

All the tested extracts were found to be noncytotoxic or with very low toxicity on LLC/MK2 monkey kidney epithelial cells. This observation may be an indicator of their safety as drugs for mammalian organisms. Some of the plants tested in this study like *V. amygdalina* and *D. edulis* are widely used in many African countries including Nigeria and Cameroon for both therapeutic and nutritional purposes [23]. No part of *D. edulis* is known to be toxic, except the presence of some antinutrient factors such as oxalate, tannins, phytates, and trypsin-inhibitors in the seeds [24, 25].

5. Conclusions

From this study, it was observed that *Dacryodes edulis*, *Vernonia amygdalina*, *Coula edulis*, *Eucalyptus globules*, and *Cuviera longiflora* possess significant antiplasmodial activities. Both leaves and roots of *V. amygdalina* showed high antiplasmodial activity with no cytotoxicity. The results obtained justify the use of these plants by traditional healers of Western Cameroon. Further investigations on some of these plants, especially including bioassay-guided fractionation of extracts from *D. edulis*, *V. amygdalina*, and *Coula edulis*, are likely to yield new antimalarial drug leads.

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