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Phytochemical screening, antiplasmodial activity against multi-drug resistant parasites, cytotoxicity and antioxidant profiling of extracts from *Fagara macrophylla* and *Eremomastax speciosa*

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Abstract

Objective: The continuous spread of drug-resistance underlies the urgent need for new drugs against this disease. Considering the serious implications of oxidative stress in the malaria pathology, the present work was prompted, aiming at investigating the antioxidant and anti-plasmodial activity of *Fagara macrophylla* (Rutaceae) and *Eremomastax speciosa* (Acanthaceae), two Cameroonian medicinal plants popularly employed in handling malaria and several degenerative diseases.

Methods: The anti-plasmodial activity was assessed using the semi-automated method of Desjardins with the parasite lactate dehydrogenase assay (pLDH) as parasite quantification system, whereas the cytotoxicity was assessed on the monkey kidney epithelial cell line (LLC-MK2) using the MTT - ELISA assy. The antioxidant evaluation was done using 1,1-diphenyl-2-picrilhydrazyl (DPPH) for free radical-scavenging properties of the extracts and the Folin-Ciocalteu method in determining their phenol contents. The phytochemical screening of both extracts was equally conducted using standard methods.

Results: The results revealed a wide range of anti-plasmodial activity in both plants, with the hexane extract of *F. macrophylla* stem bark showing the highest activity (IC_{50} = 4.94 µg/mL), followed by the methanol extract of *E. speciosa* leaf (IC_{50} = 8.85 µg/mL). More interestingly all the extracts with significant antimalarial activities did not exhibit any sign of cytotoxicity on monkey kidney cells even at 500 µg/mL. Extracts from both plants exhibited significant antioxidant properties as illustrated by their high phenols contents and scavenging activities.

Conclusion: A diversity of chemical families was detected from these extracts, indicating that *F. macrophylla* and *E. speciosa* could serve as good starting point for the development of new phytomedicines and/or drug molecules against malaria and degenerative diseases.

Keywords: Antimalarial, Antioxidant, Cytotoxicity, *Fagara macrophylla*, *Eremomastax speciosa*

Introduction

Malaria is the third most deathly disease today, after tuberculosis and the human immunodeficiency virus /acquired immunodeficiency syndrome (HIV/AIDS) [1, 2]. For long, drug resistance has remained the greatest challenge to malaria control and it is part of the obstacles that sapped the dream of seeing malaria eradicated in the years 1970s. So far, resistance has been fully established in three of the five Plasmodium species responsible for human malaria (*P. falciparum, P vivax* and *P. malariae*) and concerns virtually all drugs regiments currently used. Besides, it is recognized that malaria and poverty are intimately connected. The global distribution of per-capita gross domestic product shows a striking correlation between malaria and poverty and malaria-endemic countries also have lower rates of economic growth [3]. It is therefore an urgent but also a challenging needs to design and make available a new generation of more efficacious antimalarials especially affordable to poor communities of most rural endemic regions. Such a target could be reached more easily if research also takes into

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account the treatment or palliative tools usually employed by these populations to control the disease at the local level. Among these resources figure as priority traditional and herbal medicine in most developing countries. Local medicines are even preferred to modern medicines in ethnic groups such as Baka Pygmies in South Eastern Cameroon [4]. Traditional medicines are commonly sold in markets and public places or administered by healers in traditional clinics. Whole plants or parts of them are prepared and administered as oral decoction, steam baths, infusion or enema. Most remedies are a concoction of two or more plant species and solvents used include water, palm wine or oils. Health problems are often self-treated first with the popular pharmacopoeia also called self-aid or auto-medication [5-7]. *Fagara macrophylla* (Rutaceae) and *Eremomastax speciosa* (Acanthaceae) are commonly employed by Traditional Healers in several parts of Cameroon to handle a diversity of ailments. *E. speciosia* (L) Juss is also known as "Benjan-moh" in Bambui (NorthWest Cameroon), "Pan Dzem-moh" in Batcham (West Cameroon). Concoction of the areal part, together with leaves of *Pavonia burchselli* and leaves and stem bark of *Melinis minutifolia* in water or palm oil is taken orally to treat cough [8]. The water decoction of *E. speciosa* areal part is equally administered to children having intestina yeast infections and fever. For its own, *F. macrophylla*, locally known as "is used to treat diseases as stomac abscess, intestinal disorders and fevers. Leaves and stem bark are generally prepared as decoction in palm wine or water, or infusion as anema (for stomac abscess).

However most of these preparations have not yet been standardized as pharmaceutical products and are still to reach official prescription lists of antimalarial drugs. Up to June 2007, more than 200 medicinal plants have been recorded herein for their use as anti-malarials in Cameroonian folk medicine, but only one third of the plants so far identified have been tested for their acclaimed potentials [9]. It is equally imperative to standardize and improve on the qualities of the herbal products in order to develop local products that could be more affordable and available to poor communities of remote areas in endemic countries. These strategies, if pursued from drug discovery research to preclinical followed by clinical studies, are likely to reveal suitable candidate products against malaria.

The present investigation aimed at establishing the antiplasmodial, cytotoxicity and antioxidant profiles of *F. macrophylla* and *E. speciosa*, in order to assess their suitability to serve as source materials for the development of new products against malaria and other diseases exhacerbated by oxidative stress.

Figure 1: Illustrative photographs of the different plant parts used in the study
A: areal part of *E. speciosa*; B & C: Stem and Leaves of *F. macrophylla*, respectively

Materials and Methods

Collection of plant materials

Plant parts were collected in different localities in Cameroon as summarized in table 1. Species were identified at the Cameroon National Herbarium, Yaounde where voucher number was issued for each of the plant species. Table 1 describes the dates and localities where the plants were collected and figure 1 provides illustrative pictures of the different parts considered.

Preparation of crude extracts

The air-dried and powdered plant material (about 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, ethyl acetate and methanol. In each case, the mixture was then filtered with Whatman paper and concentrated to dryness using a Rotavapor system (BUCHI Labortechnik AG, Switzerland) at 40 ºC (methylene chloride), 65 ºC (methanol), 70 ºC (hexane) or 80 ºC (ethyl acetate) [10]. The crude extracts prepared were stored at 4 ºC for further use.

Phytochemical analysis

The extracts were screened for detection of different chemical families according to the methods previous described by Odebeyi and Sofowora [11]. In brief, phenolic compounds were detected and quantified using the ferrocyanide reaction;

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triterpenes and sterols were revealed by their reactivity with anhydrous acetate and sulphuric acid. Alkaloids were detected using sulphuric acid, whereas the presence of saponins revealed based on their foaming property. Tannins and flavonoids were revealed using ferric chloride and hydrochloric acid, respectively. Anthraquinones were detected in extracts by the chloroform/petroleum system, while the presence of lipids was assessed on filter paper.

Plasmodium falciparum culture and maintenance

Parasite strains

The parasite strain W2mef was kindly donated by BEI-Resources (MR4, Manassas, VA, USA), and maintained in continuous culture, with back up stored in liquid nitrogen.

Parasite culture

The laboratory strain of *P. falciparum* was grown and maintained in culture using the method of Trager and Jensen with some modifications [12-14]. All the chemicals except Albumax II (Gibco; Invitrogen, USA), were ordered from Sigma-Aldrich Inc (Germany). The cultures were monitored on a daily basis, and parasitemia assessed using both fluorescence (acridine orange) and normal light (Giemsa stain) microscopy.

Determination of anti-plasmodial activity

The anti-plasmodial screen was carried out in 96-well microtitration plates as described by Desjardins et al. with some modifications [15, 16]. The parasitaemia was measured using the parasite lactate dehydrogenase (pLDH) assay as previously described [**16**]. The drug-interaction patterns of the most active compounds were assessed as earlier described. From the results obtained with individual drugs, the two drugs were mixed at several proportions and each of these formulations then tested and their different inhibitory concentrations determined separately [16].

Following the pLDH assay, the optical densities of the microtiter plate wells were read and analyzed using the software HN-NonLin V1.1 [17] to generate log dose-response correlation coefficients and the 50%, 90%, 95% and 99% Inhibitory Concentrations (IC_{50} , IC_{90}, IC_{95} and IC_{99} respectively) for the individual replicate tests. Both anti-plasmodial activities and cytotoxicity studies were expressed as Mean ± SD from a pool of data obtained from at least two different experiments conducted in triplicate each. The different means were compared among themselves by independent samples t-test. The analysis of the results of the drug-interaction study was carried out as described previously [16, 18, 19].

Cytotoxicity study of active compounds

The cytotoxicity of the extracts and pure compounds were estimated on Rhesus Monkey Kidney Epithelial Cells (LLC-MK2 Line) as previously described [20] with some modifications [13]. The cell line was ordered from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in continuous culture.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts as described by Hotano *et al* [21] with slight modification [22]. Different concentrations (0.25, 0.5, 1 and 2 mg/mL) of each extract were prepared in distilled water, 30 µL of each solution mixed with 1 mL of ethanol solution of DPPH (0.1mM) and incubated for 30 min in the dark. At the end of this period, the absorbance was recorded at 517 nm using a spectrophotometer, and the antiradical activity of each concentration calculated as percentage reduction in DPPH concentration, with reference to the optical density at the start, as follow:

$$\%\ \text{scavenged [Free radicals]} = \left[\frac{Ao - A1}{Ao}\right] \times 100$$

where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract or standard.

The IC_{50} values were then generated by extrapolation from the curve of activity versus concentration.

Reduction potential of the extracts

In order to appreciate the reduction of potential of the different extracts, their polyphenol contents were determined using the method described of Folin-Ciocalteu as described earlier by Singleton and Rossi [23] with some modifications. In brief, 30 µL of extract of known concentration were thoroughly mixed with 10 mL of Folin-Ciocalteu (Sigma) 0.2 N and the absorbance measured at 750 nm, after 30 min incubation at room temperature in the dark. Catechine solutions in methanol at 10, 20, 30, 40 and 60 µM were used as standards.

Table 1: Summary information on the different plant parts used

No	Scientific name (Family)	Place of collection	Voucher number	Part used	Solvent	Code
1	*Fagara macrophylla* (Rutaceae)	Kribi, (South)	6166/SRF/CAM	Stem bark	Hexane	FMSh
					Methanol	FMSm
				Leaves	Hexane	FMLh
					Methanol	FMLm
2	*Eremomastax speciosa* (Acanthaceae)	Yaounde (Centre)	57295/HNC	Whole	Hexane	ESWh
					Methanol	ESWm

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Results and discussion

Anti-plasmodial and cytotoxic profiles

The *in vitro* antiplasmodial and cytotoxicity profiles of the different extracts are summarized in table 2 below. Against the multidrug-resistant W2mef parasite strains the extracts showed IC_{50} values ranging from 4.94 to 175 µg/mL. The methanol extract of *F. macrophylla* stem bark scored the highest activity, followed by the methanol extract of leaves from the same plant while *E. speciosa* showed moderate activity. Apart from the methanol extract of *E. speciosa*, none of the tested extracts showed significant sign of toxicity on monkey kidney cells.

Drug combination patterns of the most active extracts

A positive correlation was observed between the concentration of FMLm added and the activity of FMSh ($-0.960 \leq r \leq -0.812$, $P < 0.04$). Likewise, the addition of FMSh significantly affected the activity of FMLs ($-0.871 \leq r \leq -0,856$, $P < 0.001$). This shows that both the leaf and stem bark of *F. macrophylla* contain antimalarial ingredients. However, the combination patterns observed were rather additive, as reflected in the values of combination index recorded (CI=1.08 ± 0.12) and the isobologram obtained in figure 2.

Phytochemical composition

The results of the phytochemical screening of the most active extracts are summarized in table 3 below. In general, chemical classes of compounds detected include: alkaloids, anthraquinones, coumarines and essential oils in all extracts, though alkaloids were only in trace in *E. speciosa*. Phenols, anthocyanins and cathechic tannins were found exclusively in *E. speciosa* whereas flavonoids were present only in *F. macrophylla* which were the only plant not sterols.

Antioxidant activity

Free radical scavenging activity

The 50% scavenging concentration (SC_{50}) used in assessing the scavenging power of the extracts, is defined as the extract concentration that reduces half the total quantity of free radicals present in solution at the start of the assay. Figure 3 reports SC50 values of the extracts tested. SC_{50}s ranged from 20 µg/mL, for the methanol extract of *F. macrophylla* leaf (FMLm) and 114.34 µg/mL methanol extract of the stem bark from the same plant (FMSm). Intermediary values were 67.85 µg/mL (ESLm), 78.88 µg/mL (FMSh) and 100 µg/mL (FMLh) and 102.94 µg/mL for the hexane extract of the leaf from *E. speciosa*.

Iron chelating power of the selected extracts

The ability of each of the extracts to chelate iron is reported as iron chelating concentration 50% (IC_{50}) which is the concentration of extract trapping half of the total iron initially present in solution. The result showed IC_{50} ranging from 11.64 mg/mL for ESLm to 20.58 mg/mL for FMLm.

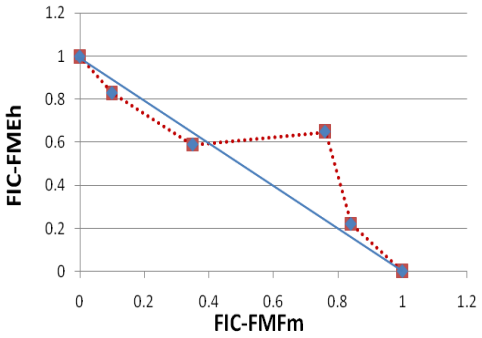

Figure 2: Isobologram illustrating the interactions between the leaf and stem bark extracts of *F. macrophylla* on W2mef *Plasmodium falciparum*

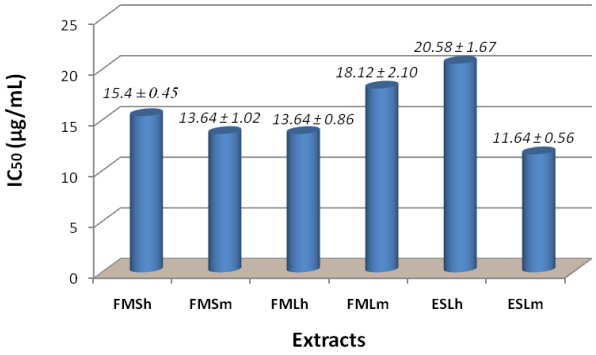

Figure 3: Iron chelating properties for the selected extracts

FMLh= Hexane extract of *F. macrophylla* leaf; FMLm= Methanol extract of *F. macrophylla* leaf; FMSh= Hexane extract of *F. macrophylla* stem bark; FMSm= Methanol extract *of F. macrophylla* stem bark; ESWh= Hexane extract of *E. speciosa* leaf; ESWm= Methanol extract of *E. speciosa*

In sub-Saharan Africa, the local populations generally resort to the use of traditionally prepared remedies. Medicinal plants also play an important socio-economic role both by fulfilling health-care needs and providing business opportunities and employment to the local people. Hit/lead discovery from plant sources is of particular interest in the tropics, targeting endemic infectious like malaria, as well diseases like diabetes and cancers which are fast expanding in Africa today [14]. A total of six crudes extracts prepared from two different plant species selected based on their frequent used in the traditional pharmacopeia, were screened for their antimalarial activities and antioxidative properties. The antimalarial activity was evaluated against the W2mef multidrug-resistant *Plasmodium falciparum* strains *in vitro*. According to standards set by Rasoanaivo *et al.* [24] two extracts from both parts of *F. macrophylla* exhibited good activities (IC_{50}<10

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µg/mL) against the multidrug-resistant strain of *Plasmodium falciparum in vitro*, whereas three of the six extracts (FMSm and ESWm and ESWh) were moderately active and one (FMLh) inactive. From literature search, the antimalarial potential of these medicinal plants was experimentally established for the first time, though their use in Africa traditional medicine is well documented [6, 9, 25]. *E. speciosa* is a multipurpose species plant of the Cameroonian traditional pharmacopeia. The plant was shown to possess a diversity of pharmacological activities including anti-anemic and antibacterial evidence [26]. Ndem et al [27] demonstrated the hepatoprotective effect of the ethanol extract of *E. speciosa* in rats with phenylhydrazine-induced anemia. Administration of 300 to 500 mg/kg resulted in significant increases in the haemoglobin, red blood cell count, and haematocrit, when compared with the normal and anaemic control groups. These findings together with the weak antiplasmodial activity observed in the present study may be an indication that the antianemic property of *E. spciosa* is more important than its antimalarial effect; still justifying the widespread use of this medicinal in malarial treatment in African medicine.

Table 2: Anti-plasmodial profile of extracts against W2mef *P. falciparum* and cytotoxicity *on LLC-MK2* cell line

Extract code	IC_{50} on W2mef (µg/mL)	IC_{90} on W2mef (µg/mL)	CC_{50} on LLC-MK2 (µg/mL)	Pharmacological significance
ESWh	45.27±3.58	169.60±9.01	51.451±7.5	Inactive; non cytoxic
ESWm	27.33± 2.29	115.27±23.05	22.43±4.8	Weakly active; weakly cytotoxic
FMLh	175.47±0.00	247.94±0.00	>500	Inactive; non cytoxic
FMLm	8.85±0.56	107.58±2.19	>500	Active; non cytoxic
FMSh	4.94±0.25	59.40±5.18	>500	Very active; non cytoxic
FMSm	22.11±4.97	77.85±22.34	68.08±5.2	Moderately active; noncytoxic
CQ	0.16±0.00	0.21±0.01	-	-
Gleevec	-	-	18.11±0.8	-

IC_{50} < 5 : very active ; 5 < IC_{50}<10 : active ; 10 < IC_{50}< 25 : moderately active; 25 < IC_{50}< 50 : weakly active ; IC_{50} > 50 : inactive [24].Values presented in the table are averages of 4 replicate tests. IC_{50}, IC_{90}, IC_{99} = 50%, 90%, 99% inhibitory concentration, respectively. CC_{50} < 5 : highly cytotoxic ; 5 < CC_{50}<10 : Cytotoxic ; 10 < CC_{50}< 30 : moderately active to weakly acytotoxic ; CC_{50} > 30 : Non-cytotoxic [Adapted from Malebo et al 2009, with some modificaitons].Values presented in the table are averages of 4 replicate tests. CC_{50}, = 50%, cytotxic concentration. FMLh = *n*-hexane extract of *Fagara macrophylla* leaves; FMLm = Methanol extract of *Fagara macrophylla* leaves; FMSh = *n*-hexane extract of *Fagara macrophylla* stem bark; FMSm = Methanol extract of *Fagara macrophylla* stem bark; ESWh =*n*-hexane extract of *Eremomastax speciosa* whole plant; ESWm = Methanol extract of *Eremomastax speciosa* whole plant; CQ = Chloroquine; Cleevec =Standard drug for cytotoxicity.

Table 3: Phytochemical composition of the different extracts

Chemical families	FMSh	FMLh	FMSm	FMLm	ESWh	ESWm
Alkaloids	+	+	+	+	Traces	Traces
Phenols	-	-	-	-	-	+
Triterpenes	+	+	+	Traces	-	-
Sterols	+	+	-	+	+	+
Flavonoids	+	-	-	-	-	+
Saponins	+	-	+	+	-	+
Anthocyanins	-	-	-	-	-	+
Anthraquinones	+	+	+	+	+	+
Glycosides	-	-	+	+	-	Traces
Gallenic tannins	-	-	-	-	-	-
Cathechic tannins	-	-	-	-	-	+
Coumarins	+	+	+	+	+	+
Lipids	+	+	+	+	+	+

FMLh = Hexane extract of *Fagara macrophylla* leaf; FMLm = Methanol extract of *Fagara macrophylla* leaf ; FMSh = Hexane extract of *Fagara macrophylla* stem bark ; FMSm = Methanol extract of *Fagara macrophylla* stem bark ; ESWh = Hexane extract of *Eremomastax speciosa* leaf ; ESWm = Methanol extract of *Eremomastax speciosa* leaf

+: present -: absent

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The phytochemical screening of the two extracts revealed the presence of a diversity of chemical species, among which the well-known classes of antimalarial family compounds, notably alkaloids and steroids. The results obtained for *E. speciosa* are quite similar to findings by Oben *et al* [28] who showed the presence of alkaloids, flavonoids, saponins and cathecic tannins in the aqueous extract of this plant. It was previously suggested that flavonoids could inhibit the development of Plasmodium, targeting the metabolism of L-glutamine; though the mechanism is yet to be fully elucidated [29]. Steroids have equally been identified in *F. macrophylla* as earlier as the beginning of the last century where Goodson et al 1920 isolated the anti-cancer lupeol from this plant species. The antimalarial potential of *F. macrophylla* was previously shown in Ivory Coast with the ethanol extract of the leaf exhibiting a high activity *in vitro* on the FcB1 strain of *P. falciparum*. However, the IC_{50} obtained in the present study with a more polar fraction of the sample collected in Cameroon (IC_{50} = was slightly lower than 2.3 µg/mL recorded by Zirihi et al [30] Several factors may justify this slight difference; these include the parasite strain (we used the multidrug-resistant W2mef strain instead of FcB1) and the edaphic factors and other environmental differences. The present work nevertheless underlines the potential of this species as source of new antimalarial products which could be either improved phytomedicines or pure molecules. It has the merit of demonstrating that, not only the leaves of the plant have antimalarial effects, but the stem bark is even more active than the leaf. Further investigations, including bioassay-guided fractionation of this part is therefore highly recommended. Exploring the active compound already identified from this plant, namely dihydronitidine (IC_{50} = 0.16 µg/mL, Zirihi *et al* [30] as well as the evaluation of the ability to the stem bark in developing phytomedicines from the leaves of *F. macrophylla*, deserve a particular attention. The implications of oxidative stress in malaria pathogenesis and severity have been extensively documented [31]. The antioxidant property of the two medicinal plants was therefore prompted in order to extend on their usefulness in malaria treatment. The DPPH test informs on the ability of a particular substance to react with free radicals of different types. It was shown that all the extracts tested possess antioxidant property, with the methanol extract of *F. macrophylla* exhibiting the highest activity (SC_{50} = 20 µg/mL). The scavenging power of these extracts is probably due to the presence of phenolic entities [32, 33] like flavonoids, tannins and coumarines detected in them from the phytochemical screening. Ferrous ions are actively involved in the production of hydroxyl and superoxide radicals, through the Haber-Weiss reaction [34]. The chelating property of the extracts could be very instrumental in inhibiting this ion-dependant process [35]. The malaria parasite relies essentially on hemoglobin to cover his need in amnio acids. By degrading the host hemoglobin, iron is released through hem cleavage, thus increasing the risk of oxidation and formation of free radicals. It has been demonstrated that iron-chelating agents could seriously interfere with several metabolic processes of the parasite, including DNA synthesis, carbohydrates and protein metabolism by blocking the proteolytic cleavage of hemoglobin and the electron transport chain in the red blood cell [36].

More interestingly, all the extracts with highest antimalarial activities did not exhibit any sign of cytotoxicity on monkey kidney cells even at 500 µg/mL. Several investigations have been carried out on the toxicity of products from these two medicinal plants, though findings are somehow contradictory. Form a study by Siwe *et al* [37], the water extract of *E. speciosa* administered by oral route at doses as high as 2000mg/kg did not produce death or any sign of toxicity in the rats. These findings were confirming those of Okokon *et al* [26] who also found no signs of writhing and body weakness following acute intake of the extract of *E. speciosa* through intraperitoneal route. Furthermore, subacute toxicity investigations by Siwe and co-workers revealed that no adverse clinical sign or toxicity sign or death throughout the treatment duration of 28 d in the rats. The authors concluded that the water extract of the aerial parts of *E. speciosa* could be considered safe based on the above investigations. The high safety margin of the extract through oral route justifies its widespread use by traditional healers.

Combining the most active extracts instead revealed additivity pattern, indicating the absence of drug interaction among the constituents of the two extracts. It could therefore be beneficial to use both plant parts in combination, as far as their effects would be added in the mixture.

Conclusion

The present study confirmed that Cameroonian medicinal plants *F. macrophylla* and *E. speciosa* could serve as good starting point for the development of new phytomedicines and/or drug molecules against malaria and other degenerative diseases. Further investigations, including *in vivo* activity and pharmacodynamics/pharmacokinetics profiling of products from these plants are therefore highly recommended.

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