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In vivo Efficacy and Toxicity Studies on *Erythrina senegalensis* and *Khaya ivorensis* Used as Herbal Remedies for Malaria Prevention in Cameroon

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Authors' contributions

This work was carried out in collaboration between all authors. Author RNT performed the experiments, analyzed the data, managed the literature searches and wrote the first draft of the manuscript. Authors GUD and SRY managed the analysis of the study. Author LL designed the study and performed the statistical analysis. Author GL designed the study and corrected the manuscript. Author AH wrote the protocol, designed the study and corrected the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: The study aimed at assessing the *in vivo* anti-plasmodial activity of aqueous extracts from *Erythrina senegalensis* and *Khaya ivorensis*, two plants used traditionally as bark decoctions in Cameroon to prevent and cure malaria.
Methodology: The antiplasmodial activity of aqueous extracts of *E. senegalensis* and *K. ivorensis* was investigated using a murine malaria model (*Plasmodium berghei* / *Anopheles stephensi* / BALB/c mice), applying a protocol for assessing the prophylactic potential of the remedy. Treatments were administered orally to BALB/c mice for 9 days at doses of 200 and 400 mg/kg/day. Mice were challenged on day 3 of treatment by

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exposure to *P. berghei* infected mosquitoes. The impact on parasitaemia was assessed on thin blood smears prepared on day 7 after exposure to infective bites.
The acute toxicity of the plant extracts was tested according to the guidelines of the Organization for Economic Co-operation and Development (OECD guidelines 423).
Results: The plant extracts showed antiplasmodial activity, reducing parasitaemia by 40.4% to 56.3%, according to the extract. In particular, a combination of the two extracts at the dose of 100 mg/kg each provided a reduction of parasitaemia in treated mice by more than 50%, as compared to controls. The extract of *E. senegalensis* when used alone at 200 mg/kg/day reduced the parasitaemia by 40.3% +/- 7.2%, doubling the dosage increased parasite suppression to 56.3% +/- 5.1%.
Toxicity studies yielded comforting results: up to a dosage of 2000 mg/kg no mortality occurred in treated mice. Also, animals treated during the antiplasmodial experiments did not reveal signs of toxicity and remained in good conditions up to the end of the experiments.
Conclusion: The results suggest that the combination *of E. senegalensis* and *K. ivorensis* could be a valid plant combination for the preparation of a standardized, effective and affordable remedy against malaria, in particular for Cameroonian communities with limited access to modern drugs.

Keywords: Antiplasmodial activity; acute toxicity; Erythrina senegalensis; Khaya ivorensis; validation.

ABBREVIATIONS

ANOVA: Analysis of variance; AOT: acute oral toxicity; GHS: Globally Harmonized Classification System; iRBC: infected red blood cells; LSD: least-significant difference; mG: microgametocytes; MG: macrogametocytes; NHC: National Herbarium of Cameroon; OECD: Organization for Economic Co-operation and Development; U.S.EPA: United States Environmental Protection Agency; WBC: white blood cells; WHO: World Health Organization.

1. INTRODUCTION

Plasmodium falciparum malaria is widespread in Cameroon. In 2011 it caused an estimated number of 429,700 cases and about 3,800 deaths despite pharmacological and vector control measures implemented according to WHO guidelines since 2004 [1].

Nowadays, Cameroonians of all ranks and backgrounds use traditional medicine for economic reasons - modern health service accessibility is often limited - as well as for personal preferences. The 2002 annual report of the Ministry of Public Health confirmed that the economic crisis and the absence of a functional social security system have created a strong return to traditional health services. About 7% of the average household health budget goes to traditional medicines. Nearly twice as many people from poor households rely on traditional medicine as do people from rich households [2]. Although incorporating traditional medicine into the national health system is not a priority in Cameroon, WHO recognizes traditional medicine as a vital health-care resource [3].

In Cameroon, ethnobotanical studies have identified more than 48 antimalarial plants [4]. *E. senegalensis* and *K. ivorensis* stem bark decoctions are frequently reported as being used

for malaria treatment and prevention in Western and Central Cameroon. Given the wide use of the two plants, it is important that their claimed efficacy is investigated and that possible toxic effects are assessed.

E. senegalensis DC. (coral tree) is commonly grown in West Africa as an ornamental plant and one of the oldest known African medicinal plants [5]. The bark and roots are used against stomach disorders and wounds. In Cameroon and Nigeria, preparations from different parts of *E. senegalensis* are used orally, in body baths or as fumigations to treat malaria and fevers, dysmenorrhoea, pneumonia, cough, onchocercosis, snake bites, gastrointestinal disorders, inflammation, backache, nose bleeding, prostate, dizziness, jaundice and veneral diseases [6]. Stem bark preparations of this tree are traditionally used by the Bamun population (Western Cameroon tribe) against liver disorders [7].

The bark, roots and leaves of *K. ivorensis* A. Chev. (African Mahogany) are traditionally used for the treatment of various types of ailments including several infectious diseases reviewed in a recent *in vivo* study which provided evidence on the efficacy of a combined decoction of *K. ivorensis* and *A. boonei* as prophylactic antimalarial in Centre Cameroon [8].

Several *in vitro* and *in vivo* murine model studies have evidenced the antiplasmodial activity of extracts derived from these plants against asexual blood stages [9,10]. However, *in vivo* preventive activity has rarely been explored.

This study aims at evaluating the antiplasmodial activity of plant extracts derived from *E. senegalensis* and *K. ivorensis*, focusing on their prophylactic potential and their possible additive/synergistic effects using a malaria murine model. In addition, the safety of single and combined plant extracts was assessed.

2. MATERIALS AND METHODS

2.1 Plant Extracts Preparation

K. ivorensis stem bark material was collected during the dry season in the central region of Cameroon (Mbalmayo) and *E. senegalensis* stem bark material was collected in Dschang (March 2008). The plants were identified by a plant taxonomist, Mr. Victor Nana and voucher specimens were deposited (Nº 53957 NHC for *K. ivorensis* and Nº 50119 NHC for *E. senegalensis*) at the National Herbarium of Cameroon (Yaounde).

Fresh pieces of *E. senegalensis* and *K. ivorensis* stem barks were air dried under shade in the laboratory for 2 weeks and reduced to powder using an electric mill. The powders were stored in dark and dry conditions.

Decoctions were prepared by boiling 50 g of powdered plant material in 1 liter of distilled water for 15 min. The solution was then centrifuged (10,000 rpm for 10 min) using a RC-5 Superspeed Refrigerated Centrifuge DUPONT Instruments SORVALL, filtered, freeze-dried, and the dry extracts obtained stored in glass tubes at - 20ºC. A combination extract was prepared by mixing the lyophilized powder obtained from the two aqueous extracts, following the traditional recipes. The yield of the aqueous extracts of *E. senegalensis* and *K. ivorensis* measured as g of extract for 100g of dry powder material was 19% and 13% respectively. Traditionally, about 75g bark material of each of the two plants are mixed and boiled in a

volume of “one bottle” (~1500L) of water for 15 min. Then, an adult drinks three glasses of the decoction daily for one week.

2.2 Murine Malaria System

The chloroquine-sensitive *Plasmodium berghei* ANKA strain was used to assess *in vivo* anti-malarial activity. The parasite strain was maintained by acyclic passages from infected to healthy BALB/c mice. Cyclic passage of *P. berghei* parasite to *Anopheles stephensi* mosquitoes was obtained by feeding 3-5 days old *Anopheles* females on gametocytaemic mice. Mice were anaesthetized using a mixture of 1 volume of Rompun (xylazin 2%), 1 volume of Prequillan (acepromazine, 10 mg) and 3 volumes of Phosphate-Buffered Saline (PBS), pH 7.2, at 0.05 ml/10 g body weight.

Female and male BALB/c mice 8 - 12 weeks old and weighing 19 - 25 g were used for this study. The experiments were carried out in the animal facility of the University of Camerino (Italy). The experimental animal room was maintained at a temperature of 22ºC (+/-3ºC), 30-70 % relative humidity and 12 h photoperiod. Animals were fed with standard rodent pellets (Mucedola s.r.l., Milano, Italy) and provided with water *ad libitum*.

Mosquito rearing from the larvae to adult mosquitoes took place in the insectary at 28ºC (+/-2ºC) and 80% (+/- 10%) humidity. Female mosquitoes infected with *Plasmodium berghei,* through a blood meal on gametocytaemic mice, were kept at 19 (+/- 1ºC) for the whole duration of the sporogonic cycle. On day 10 after the blood meal, mosquitoes were dissected to evaluate their oocyst positivity. On day 21, after completion of the sporogonic cycle, females were divided into groups of 20 each and transferred to the 28ºC chamber. The following day, these mosquitoes were used for the challenge of the experimental mice. Salivary glands of 10 - 20 mosquitoes were dissected to confirm the presence of sporozoites.

2.3 Assessment of *in vivo* Antiplasmodial Prophylactic Activity of Plants Extracts

The antiplasmodial activity of the aqueous extracts was assessed in the mouse model at dosages corresponding approximately to the amount of plant material taken up by malaria patients according to the traditional medicine recipes. Groups of six mice each received orally one of the following aqueous extracts: *E. senegalensis* (E) at 200 mg/kg/day (E200) and 400 mg/kg/day (E400), 1:1 mixture of *E. senegalensis* and *K. ivorensis* extracts (EK) at 100 mg/kg/day each (EK100+100).

The powder extracts were dissolved in distilled water before the gavages. Test solutions were administered orally twice a day (every 12 hours using half of the daily dosage) in volumes of 200 µl for a period of 9 days. Control animals were treated similarly with distilled water. On the third day of treatment, experimental mice were individually infected through the exposure to 20 bites of infective mosquitoes, 1 hour after the first treatment of that day. The number of mosquito bites was estimated, counting the fed mosquitoes at the end of the infection. One day after the last treatment (Day 7 post-infection), a puncture was made on the mouse tail and a drop of blood collected for the Giemsa-stained thin blood smears. Parasitaemia was determined to estimate the impact of treatments on asexual blood stages by counting the number of parasitized erythrocytes for 3000 red blood cells. The average percent reduction of parasitaemia was calculated as -

$$\% \text{ reduction} = [(C - T) / C] * 100$$

Where C is the arithmetic mean of parasitaemia in the control group and T the arithmetic mean of parasitaemia in the treated group. Almost each experiment was performed in duplicate.

The ratio between the number of gametocytes and the number of infected red blood cells per µl of blood was also assessed to estimate the effect of treatments on gametocytaemia [11]. The concentration of infected red blood cells per µl blood (iRBCs/µl) was estimated in relation to the counts of white blood cells (WBC), according to the following formula:

iRBCs/µl = [(iRBC in 25 microscopic fields at 1000x magnification)/ (WBC counted in 25 fields at 1000x magnification)] *13,000

Considering 13,000 the average concentration of WBC/µl of BALB/c mouse blood.

2.4 Evaluation of the Acute Oral Toxicity

The acute oral toxicity (AOT) is measured as the mortality caused within a short time by the oral administration of a substance, either given in a single dose or in multiple doses within 24 hours and is expressed as LD_{50} (median lethal dose), the dose that causes death in 50% of the animals.

The AOT of *K. ivorensis* has already been measured in our laboratory, using the method of Thompson and Weil (12). Four geometrically increasing doses (factor 1.5) were chosen: 823.5 mg/kg, 1235.25 mg/kg, 1853 mg/kg and 2779.5 mg/kg [8].

In order to reduce as much as possible the number of experimental mice, acute toxicity assessments for the aqueous extracts of *E. senegalensis* and its combination with *K. ivorensis* were performed according to the OECD 423 guidelines [13]. This protocol used one or two groups of three animals each at a fixed dose of 2000 mg/kg, depending if the mice of the first group died or survived.

Groups of six, 8 to 10 weeks old, male BALB/c mice received a single oral treatment using a stomach tube. Three animals were used the first day, followed by another three 48 h later. Animals were fasted over night prior to substance administration and food was withheld for a further 1-2 h after the substance had been administered. The animals were observed after the treatment for the first 30 minutes, then periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter for a total of 14 days for any sign of toxicity (discomfort, morbidity, changes in the skin and fur, eyes, somatomotor activity and behavioural pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual weights of animals were measured shortly before the test substances were administered and weekly thereafter.

2.5 Statistical Analysis

Quantitative data were expressed as arithmetic mean values ± standard deviation. To compare parasitaemia and gametocytaemia levels, the independent samples Student's t-test was used. For other results, one-way analysis of variance (ANOVA) followed by the least-significant difference (LSD) post hoc tests were applied.

3. RESULTS AND DISCUSSION

3.1 *In vivo* Antiplasmodial Activity

The administration of the *E. senegalensis* extract at 400 mg/kg yielded a reduction in parasitaemia of 56.3% +/- 5.1%, corresponding to a mean parasitaemia value of 5.2% +/- 0.9% in the treatment group, compared to 11.8% +/- 1.7% in control group (Table 1).

At a dosage of 200 mg/kg, the preparation reduced the parasitaemia by 40.3% +/- 9.3% (Table 1). The observed antiplasmodial activity was lower than that recorded with the 400 mg/kg/day dosage, evidencing a dose-dependent activity.

This result is consistent with those on *in vitro* and *in vivo* antiplasmodial activities of *E. senegalensis* that have been reported in the literature: the aqueous extract of *E. senegalensis* administered orally to mice indicated suppressive activities of 16.5% and 23.2% compared to 95.8% for chloroquine [9]. The relatively stronger activity in our study is probably due to high doses of extracts given to mice (200 and 400 mg/kg) compared to 50 and 100 mg/kg in the cited study, as well as to the different experimental procedures adopted by the authors (suppressive and curative tests versus prophylactic test in our study).

In vitro screening for antiplasmodial activity of ethanolic and methanolic extracts of *Erythrina senegalensis* conducted by a Nigerian researchers group showed moderate to good activities against the K1 multiresistant *P. falciparum* strain, with IC_{50} values of 99.7 and 1.82 µg/ml respectively [10,14].

Interestingly, an aqueous extract of the stem bark of *E. senegalensis* was found to induce anti-inflammatory [14], analgesic and antipyretic effects in rats [9,14]. Such immuno-modulatory effects might be associated to the anti-malarial, febrifuge effects reported by the populations practicing the treatment with these traditional remedies.

The combination of *E. senegalensis* and *K. ivorensis* was found to possess considerable antiplasmodial activity in the mice. The 7 days administration of a combination of the two extracts at a dosage of 100 mg/kg each resulted in a parasitaemia reduction of 56.3 +/- 4% with parasitaemia values of 5.0% +/- 0.9% and 5.3% +/- 0.8% in the treatment groups of the two experiments, significantly lower than those observed in the controls (Table 1; Fig. 1).

In a previous study, the aqueous extract of *K. ivorensis* at the dose of 400 mg/kg resulted in a parasitaemia reduction of 35.5% +/- 12.7% [8]. This activity was attributed to the presence of chemical compounds such as anthocyanins, flavonoids, steroids, tannins and the limonoid gedunin [15]. The molecule gedunin has shown strong *in vitro* antiplasmodial activity with an IC_{50} value of 0.72 µg/ml [16]. The phytochemical analysis of *E. senegalensis* stem bark extracts revealed the presence of tannins, alkaloids and cardiac glycosides [17]. Good absorption and bioavailability associated to positive interactions between these compounds may explain the observed impact of the combination.

The similar activity displayed by the combined extract at the dose of 100mg/kg +100mg/kg compared to the single extract at the highest dose (400 mg/kg) encourages the use of *E. senegalensis* in combination. In addition, the combination allows for a relatively low dose

intake of *E. senegalensis* that may help to avoid early appearance of toxicity and attenuate possible side effects of the plant [18].

Table 1. Effect of *E. senegalensis* and *K. ivorensis* stem bark extracts on *Plasmodium berghei* ANKA strain.

Treatment[1]	Dose (mg/kg/day)	Experiment[2]	Nº infective bites per mouse	% Mean parasitaemia ± S.D.
E	200	1	16.3	6.0 ± 1.8 *
		2	19.8	7.3 ± 1.8 *
E	400	2	19.0	5.2 ± 0.9 *
EK	100+100	2	18.8	5.0 ± 0.9 *
		3	19.5	5.3 ± 0.8 *
Control		1	17.2	10.5 ± 2.7
		2	19.7	11.8 ± 1.7
		3	19.7	11.8 ± 1.7

[1]*E, aqueous extract of E. senegalensis; EK, mixture of E. senegalensis and K. ivorensis extracts.*
[2]*Each number represents one independent experiment involving treatment and control groups of six mice each.*
*Test extracts: significant from control, * P < 0.05*
Mean ± S.D. = Mean values ± Standard deviation of mean parasitaemia of six experimental mice

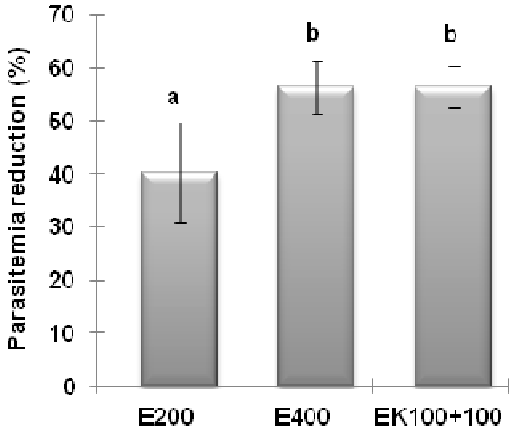

Fig. 1. Parasitaemia reductions by oral administration of various concentrations of *E. senegalensis* stem bark decoction and its combination with *K. ivorensis*
Extracts were administered at various doses to groups of mice (n=6) for 9 days. Plasmodium berghei challenge was carried out on day 3 of treatment. Giemsa smears were evaluated on day 7 post-challenge.
E200 and E400, aqueous extract of E. senegalensis at 200 and 400 mg/kg dosages; EK100+100, mixture of E. senegalensis and K. ivorensis extracts at 100 mg/kg each.
[a,b]*Test extracts: significant difference between treatments. One way ANOVA followed by LSD test.*
Mean ± S.D. = Mean values ± Standard deviation of mean parasitaemia reduction of six experimental mice.

In this study, the combination of *E. senegalensis* and *K. ivorensis* evidenced a higher antimalarial activity (56.3% reduction) than that observed with the combination of *K. ivorensis* and *A. boonei* (43.6% reduction) at the same dosage and using the same solvent

of extraction [8]. The extract of *E. senegalensis* might be considered as a good candidate for substitution of *Alstonia boonei* because of its botanical availability, the wide distribution of the plant in the country, its safety, efficacy and its curative property against both malaria and hepatitis disease[19]. In the context of herbal medicine, even low levels of synergy between plants are still useful as they contribute to the overall effect of the remedies [18].

Our findings support the validity of the combination approach adopted by traditional medicine systems and encourage its application for the improvement of traditionally used monoherbal remedies [20].

The statistical comparison of the proportions of macro- and micro-gametocytes on infected red blood cells (iRBCs) was not able to reveal any difference between treatment and control values (Table 2), meaning that, neither *E. senegalensis* alone or in combination with *K. ivorensis* had an impact on gametocyte development.

As for a study on "N'Dribala" and "Saye" (combination remedy between *Cochlospermum planchonii* roots, *Cassia alata* leaves and *Phyllanthus amarus* whole plant), the overall reduction of micro and macrogametocyte numbers is just reflecting the suppressive effect of the extracts on reproduction of the asexual blood forms [11].

This study supports the absence of extract effects targeted to gametocytes but only an effect on asexual stages of parasites.

Table 2. Effect of *E. senegalensis* and *K. ivorensis* stem bark extracts on gametocytaemia

Treatment[a]	Experiment[b]	MG/µl x10³	mG/µl x10³	MG/iRBCs(%)[c]	mG/iRBCs(%)[c]
E200	1	3.32 ± 5.07	3.77 ± 2.43	0.44 ± 0.50	3.28 ±1.88
	2	1.66 ± 1.22	3.57 ± 2.30	1.17 ± 1.21	2.06 ± 1.04
E400	2	0.72 ± 1.31	8.10 ± 3.97	0.38 ± 0.66	3.22 ± 1.62
K400	3	14.3 ± 7.4	1.66 ± 2.25	5.41 ± 1.45	0.49 ± 0.61
EK100+100	2	1.87 ± 2.15	7.82 ± 11.3	0.39 ± 0.44	2.09 ± 2.53
	4	1.70 ± 1.52	4.88 ± 3.38	0.71 ± 0.73	1.82 ± 1.55
Control	1	7.73 ± 4.58	3.63 ± 2.19	0.96 ± 0.66	3.97 ± 1.53
	2	1.49 ± 0.52	7.26 ± 3.70	0.59 ± 0.35	2.57 ± 0.94
	3	23.0 ± 16.5	3.23 ± 1.21	5.74 ± 5.05	0.90 ± 0.63
	4	1.49 ± 0.52	7.26 ± 3.70	0.59 ± 0.35	2.57 ± 0.94

[a]*K400, aqueous extract of K. ivorensis at 200 mg/kg; E200, E400, aqueous extracts of E. senegalensis at 200 mg/kg and 400 mg/kg; EK100+100, mixture of E. senegalensis and K. ivorensis extracts at 100 mg/kg each.*
[b]*Each number represents one independent experiment involving treatment and control groups of six mice each.*
[c]*Average number of gametocytes per 100 IRBCs.*
Mean ± S.D. = Mean values ± Standard deviation of mean micro/macrogametocytes of six experimental mice.

3.2 *In vivo* Acute Toxicity Test

Oral administration of a single dose of *E. senegalensis* aqueous extract proved to be safe in the acute toxicity test as well as its combination with *K. ivorensis* at the dose of 2000 mg/kg (Table 3). No mortality and visible signs or symptoms of toxicity were observed in any of the

treated animals for the first 24 h, during which however, few mice expressed some signs of prostration or excitability (jumping or grooming movements). No visible organ dysfunctions, no convulsion, no salivation, no diarrhea as well as no other signs of morbidity were observed during the first 4h until the end of the 24h of observation.

Up to the OAT threshold of 2000 mg/kg, our plant extracts can be defined as practically non toxic according to U.S.EPA criteria (United States Environmental Protection Agency) [21]. For comparison, LD_{50} value up to 2000 mg/kg was previously reported for an aqueous extract of *K. ivorensis* [8] and LD_{50} value of 5000 mg/kg for a chloroformic extract of *E. senegalensis* [22] respectively.

Moreover, no late toxicity was observed up to the end of the experimental period (day 14) and mice remained in a general good condition. Our findings are consistent with reports on the absence of mortality or significant behavioural changes in mice, receiving orally *E. senegalensis* aqueous stem bark extract and, observed up to 14 days after treatment with increasing doses of 1.25 g/kg to 12.5 g/kg [23].

Mice grew normally during the fourteen days of the experimental period (Table 3) and body weight measurements in mice treated with 2000 mg/kg of the extracts on day 0 (before treatment), on day 7 and on day 14, showed a weight increase of 3 g (Table 3), corresponding to that of healthy male BALB/C mice at the age of 8 to 10 weeks [24].

As the expected OAT level is between 2000 mg/kg and 5000 mg/kg in this study, we can classify our formulations at category 5 of the GHS (Globally Harmonized Classification System). Nevertheless, further investigations are planned to confirm the LD_{50} value at 5000 mg/kg (unclassified preparation dose) as recommended in the OECD 423 guidelines [13].

Table 3. Body weight measurements of mice treated with *E. senegalensis* and its combination with *K. ivorensis* stem bark extract at doses of 2000 mg/kg

Plant extract	Body weight (g) ± S.D.			Body weight gain (g)
	Day 0	Day 7	Day 14	
Erythrina senegalensis	19.0 ± 2.1[a]	21.5 ± 1.7[b]	22.7 ± 1.8[b]	3.7 ± 0.6
Erythrina-Khaya	20.3 ± 0.9[a]	23.2 ± 1.2[b]	23.7 ± 1.3[b]	3.4 ± 1.0

[a,b]*Test extracts: significant difference between weekly* measurements*. One way ANOVA (LSD test)*. *Mean ± S.D.=Mean values ± Standard deviation of body weight measurements of six experimental mice.*

In summary, our preclinical studies on the *Erythrina-Khaya* combination provided evidence for the remedy's efficacy and safety, and support its traditional use for the management of malaria, in particular in settings where access to modern drugs is limited.

4. CONCLUSION

The antiplasmodial activity and the wide dose interval between the therapeutic dosage (200 mg/kg) and the toxic dosage (>2000 mg/kg) exhibited by the combination of the two plants in the murine model argue in favor of their use as antimalarial remedy for preventive and - in the absence of modern drugs - for curative purposes. The results encourage also further studies to gain insight on the mechanisms underlying combinatory effects.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, in full compliance with the European Union guidelines for experimentations on laboratory animals (Council Directive 86/609/EEC of 24/11/86) and the Italian Directive 116 of 27/10/92 on the “use and protection of laboratory animals” (licence no. 125/94A, issued by the Italian Ministry of Health). All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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