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Antimalarial efficacy and toxicity evaluation of 80% ethanol extracts from the stem bark of *Enantia olivacea, Garcinia punctata* and *Massularia acuminata*

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ABSTRACT

The aim of this study was to evaluate the antimalarial efficacy and the acute toxicity of the 80% ethanol extracts of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* stem barks. The *in vivo* antimalarial efficacy of these extracts was investigated alone or in combination by the 4-day suppressive test. To assess the acute toxicity, mice were treated with a single oral dose of each individual (2,000 mg/kg) and combined extract (≤ 1,200 mg/kg). During 7 days, the animals were observed for any clinical signs of toxicity, changes in body weight and mortality. At 200 mg/kg, *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* extracts resulted, respectively, in 57.2 %, 45.3 % and 32.6 % reduction of parasitemia in mice. The combination therapy of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* (50 and 200 mg/kg of each extract) showed increased protection and survival rate associated with a significant delay of recrudescence compared with each monotherapy. No acute toxicity was observed in all the treated mice, and the 50 % lethal dose of the combined extract was assumed to be > 1,200 mg/kg. These results can partly support the use of these three plant parts for the treatment of uncomplicated malaria in Congolese traditional medicine.

Keywords: Congolese medicinal plants, combination therapy, *Plasmodium berghei*, hemolysis assay, acute toxicity

INTRODUCTION

Malaria is a major parasitic disease that continues to be one of the greatest causes of morbidity and mortality in the world. In 2017, ninety-one countries were malaria-endemic including the Democratic Republic of the Congo (WHO, 2017). Located in Central Africa, the Democratic Republic of the Congo

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(DR Congo) has annually an estimated 12 million malaria cases and nearly 100,000 deaths that account for approximately 10-15% of the estimated global malaria deaths (PNLP, 2011; WHO, 2017). The majority of deaths occur in young children (6 months to 5 years of age) and pregnant women. The elevated malaria incidence in this country is due to the high prevalence of *Plasmodium falciparum* (92.1% as monoinfection), the most dangerous of the human malaria parasites (Taylor et al., 2011; Messina et al., 2011; WHO, 2017). The wide presence of *Anopheles gambiae*, the long-lived and human blood-feeding vector as well as environmental conditions for prolific breeding also contribute to the increased rates of malaria infection in the DR Congo (PNLP, 2011; Memvanga et al., 2015).

Since 2001, the Congolese government has adopted artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *falciparum* malaria as recommended by the World Health Organization guidelines (WHO, 2010). However, the high cost of ACTs limits their accessibility to a large Congolese population. The multiple adverse effects due to the partner drug also reduce the adherence to ACTs leading to exposure of parasites to low doses of drugs, which may facilitate treatment failures and development of resistance. Moreover, ACT treatment is not recommended for children weighing less than 5 kg or women in their first trimester of pregnancy (Eastman and Fidock, 2009).

Hence, there is a need to discover new affordable, effective and less toxic antimalarial drugs that could be more beneficial for the health care management of malaria including pediatric and pregnancy-associated malaria. For this purpose, plants used in Congolese traditional medicine represent an important source of new antimalarial phytomedicines (Memvanga et al., 2015). Therefore, we focused this study on three plants (*Enantia olivacea* stem bark, *Garcinia punctata* stem bark, and *Massularia acuminata* stem bark) that are used singly or in combination by some Congolese traditional healers for the treatment of presumed malaria or malaria-like symptoms (Musuyu Muganza, 2006).

Since the *in vitro* antiplasmodial activity of the stem bark of these plants was previously determined (Muganza et al., 2012; Maloueki et al., 2015), the present study aimed at evaluating the *in vivo* antimalarial efficacy of their 80% ethanol extracts either alone or in combination in *Plasmodium berghei*-infected mice. The hemolytic activity of the three extracts was also assessed. Finally, the toxicological evaluation of the individual and combined extracts was carried out through acute toxicity profiling.

MATERIALS AND METHODS

Plant material and extraction procedure

Plant samples were collected in November 2011 in the Salonga National Park, Maï-Ndombe district, Bandundu province, DR Congo. Voucher specimens were deposited at the Institut National d'Etudes et de Recherches en Agronomie (INERA) of the University of Kinshasa (DR Congo) where the three plants were authenticated (Table 1).

The plant materials were air-dried protected from light and reduced to powder. These dried powders were then macerated with 80% ethanol (ratio of 1:10, w/v) at room temperature for 24 h under permanent shaking. The filtrates were evaporated to dryness *in vacuo* at 40 °C. All the extracts obtained were weighed and their yield calculated (Table 1).

Chemicals and reagents

Triton-X 100 and Phosphate buffered saline were purchased from Sigma-Aldrich (Diegem, Belgium) and Invitrogen Gibco (Merelbeke, Belgium), respectively. Quinine was supplied by Pharmakina (Bukavu, DR Congo). Ethanol and all the other chemicals were obtained from Merck (Darmstadt, Germany).

In vitro erythrocyte toxicity study

To assess a possible damage caused by the extracts on the erythrocyte membrane, a hemolysis test was performed as previously described (Memvanga et al., 2013). Briefly, the human erythrocyte suspensions with hematocrit values of 4 % were incubated under agitation at 37 °C for 30 min with 1 % (v/v) of ethanolic solutions of each extract (0-20 mg/ml). Ethanol was used as a negative control and Triton X-100 (1 %, w/v) as a positive control. Phosphate buffered saline was used as standard. After centrifugation (2,000 × *g*, 5 min, 37 °C), the absorbance of the hemoglobin released in the supernatants was measured by spectrophotometric analysis at 540 nm. The percentage of hemolysis was determined by the following formula:

$$\% \text{ Hemolysis} = \frac{\text{ast - anc}}{\text{apc - anc}} \text{ x } 100$$

where ast = absorbance of sample-test, apc = absorbance of positive control, anc = absorbance of negative control.

The hemolytic activity of each crude extract was tested in triplicate.

***In vivo* antimalarial efficacy**

To evaluate the *in vivo* antimalarial efficacy of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* extracts as well as combined extracts, the 4-day suppressive test was performed as described previously (Memvanga and Préat, 2012; Memvanga et al., 2013). Briefly, on day 0, NMRI mice weighing 25 g on average and aged 6–8 weeks were inoculated intraperitoneally with 1×10^7 *Plasmodium berghei* parasitized erythrocytes. The test mice were then randomly divided into groups of seven. Four hours after infection, the mice were treated orally with 0.1 ml of a single daily dose of each extract dissolved in 50 % ethanol (200 mg/kg) for 4 consecutive days. Two others groups of mice received orally 0.1 ml of a combined extract of *Enantia olivacea* + *Garcinia punctata* + *Massularia acuminata* (50 and 200 mg/kg of each extract, respectively). The control groups received quinine (10 mg/kg, positive control) or 50 % ethanol (negative control). The final group of mice was infected but not treated.

Antimalarial efficacy was assessed by the parasitemia level, the activity, the mean survival time and the survival rate of the mice for up to four weeks following inoculation. The parasitemia level was monitored on day-4 post-infection by counting, in random fields of the microscope, the number of parasitized erythrocytes per 1,000 erythrocytes. The average percent antimalarial activity (equal to the percent suppression) was determined according to the following formula (Tona et al., 2001):

$$\text{Activity} = 1 - \frac{\text{Mean parasitaemia of treated group}}{\text{Mean parasitaemia of control group}}$$

All the animal experiments were approved by Ethical Committee of animal use of the University of Kinshasa.

***In vivo* acute toxicity assay**

For the assessment of acute toxicity, thirty-five adult NMRI mice (25 ± 3 g) were randomly divided into seven equal groups. The mice were starved for 24 h prior to the administration of 0,1 ml of a single oral dose of 2,000 mg/kg of each individual extract, a mixture containing the three extracts (400, 800 and 1,200 mg of each extract per kg body weight) or the vehicle alone (i.e. 50 % ethanol). Thirty minutes after the gavage, the mice were allowed to take food again. For the first 6h, mice were closely observed for any discomfort or signs of toxicity. During 7 days, each mouse was then monitored for body weight, toxic symptoms and mortality. All the animal experiments were approved by Ethical Committee of animal use of the University of Kinshasa.

Statistical analysis

Statistical differences between rates of parasitemia suppressions and body weights were compared by one-way ANOVA with Tukey's *post-hoc* test (with a level of significance of $p < 0.05$).

Table 1: Studied plant species.

Species Botanical names	Synonyms	Families	Local names	Voucher Numbers (INERA)	Yield of ethanol extracts (%)
Enantia olivacea Robyns & Ghesq.	*Annickia olivacea* (Robyns & Ghesq.) Setten & Maas	Annonaceae	Ikodzi konga	PROCUV 1683	9.64
Garcinia punctata Oliv.	*Garcinia dandii* De Wild. *Garcinia longiacuminata* Engl. ex De Wild.	Clusiaceae	Bosepe	PROCUV 1670	25.24
Massularia acuminata (G. Don) Bullock ex Hoyle	*Gardenia acuminata* G. Don *Pomatium dubium* G. Don *Randia acuminata* (G. Don) Benth. *Randia cacaocarpa* Wernham	Rubiaceae	Welo	PROCUV 1814	16.47

RESULTS

The yield of each prepared extract is presented in Table 1. *Garcinia punctata* extract displayed the best yield followed by *Massularia acuminata* and *Enantia olivacea* extract.

At concentrations of ≤ 200 µg/ml , i.e those taking into account the dispersion of the targeted dose in gastric medium, all the extracts were found to exhibit negligible hemolytic effect (2.1-4.5%). Triton X-100, a known hemolytic agent showed 100% hemolysis of erythrocytes while ethanol showed negligible hemolysis of the erythrocytes.

At 200 mg/kg dose regimen, oral delivery of *Enantia olivacea* ethanol extract resulted in a suppression of parasitemia of 57.2% at day-4 and a mean survival time of 13.2 days (Table 2). The 80% ethanol extract of *Garcinia punctata* (200 mg/day × 4 days, oral) reduced the parasite burden by 45.3% with a mean survival time of 10.4 days. At the same dose, the 80% ethanol extract from the stem bark of *Massularia acuminata* exhibited a much lower antimalarial efficacy (32.6%) compared with those of *Enantia olivacea* and *Garcinia punctata*. Interestingly, the survival rate obtained in each extract-treated group was higher than that from the untreated group ($p < 0.05$).

The *in vivo* experiment indicated that the co-administration of *Enantia olivacea* (50-200 mg/kg), *Garcinia punctata* (50-200 mg/kg) and *Massularia acuminata* (50-200 mg/kg) decreased significantly blood parasitemia (59.6-64.5% chemosuppression) compared with each monotherapy groups ($p < 0.05$, Table 2). Unfortunately, at 50 mg/kg and 200 mg/kg, this combination was not fully effective in suppressing recrudescence thereby resulting in a survival rate of 43% and 57% at day-14 and a mean survival time of 15.2 days and 18.4 days, respectively.

In this preliminary toxicity study, no mortality was observed among all the mice treated orally with a single dose of either the individual or the combined extracts. Within the first 6 hours, mobility and aggressiveness of some treated animals were reduced. Interestingly, in the following hours and days, no remarkable behavioral changes (dizziness, motion, reaction to food supply, contact or noise, aggressiveness, etc.) neither external toxic effects (itching, curved tail, shivering, falling of hair, diarrhea) were observed. In addition, during the 7-days observation period, all the treated mice continued to gain body weight at a similar rate to that recorded in the untreated mice ($p > 0.05$, data not shown).

Table 2: *In vivo* antimalarial activity of extracts and mean survival time of mice.

Tested samples	Dose (mg/ kg/ day)	% Activity[a]	Number of survivals		Mean survival time (days)[b]
			Day 7	Day 14	
E	200	57.2	7/7	2/7	13.2 ± 2.8
G	200	45.3	6/7	1/7	10.4 ± 3.1
M	200	32.6	4/7	0/7	8.3 ± 2.5
E + G + M	50 + 50 + 50	59.6	7/7	3/7	15.2 ± 3.6
E + G + M	200 + 200 + 200	64.5	7/7	4/7	18.4 ± 5.1
Q	10	100	7/7	7/7	25.1 ± 2.7
50% EtOH	-	-	1/7	0/7	6.7 ± 2.3
Untreated	-	-	1/7	0/7	6.9 ± 1.1

E = 80 % ethanol extract of *Enantia olivacea* stem bark, G = 80 % ethanol extract of *Garcinia punctata* stem bark, M = 80 % ethanol of *Massularia acuminata* stem bark, Q = Quinine

[a] Average activity of formulations was determined on day 4 of treatment

[b] Mean survival time of mice determined during an observation period of 28 days.

DISCUSSION

Little is known about the use of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* in the traditional management of malaria in DR Congo and Africa. Indeed, the literature reports that the decoction of *Garcinia punctata* stem bark is mainly used against colic, gastritis, worms, diarrhea, headache, costal margin pain, cough, and syphilis while the macerate is reputed for its aphrodisiac properties as well as the treatment of splenomegaly and filiariasis (Akendengue and Louis, 1994; Noumi et al., 1998; Muganza et al., 2012). It also indicates that the stem bark powder is smoked to treat sexual weakness or applied topically against snake bites (Musuyu Muganza, 2006). Moreover, Tereshima et al. (1988) reported that, in DR Congo (ex-Zaïre), the leaves, fruits and seeds of this plant are known for their antimicrobial, antitussive, diuretic, laxative and anti-inflammatory properties (Tereshima et al., 1988).

Concerning *Enantia olivacea*, the aqueous decoction from its stem bark is drunk against intestinal worms, intestinal spasms and asthenia while the stem bark of *Massularia acuminata* is used, as an aqueous decoction, to treat liver diseases, back pain, hemorrhoids, premature ejaculation and erectile dysfunction (Yakubu et al., 2008; Muganza et al., 2012; Sangare et al., 2012). The stem bark of *Massularia acuminata* is also used as chewing stick for oral hygiene whereas the juice from its fruit is used against conjunctivitis (Gill, 1992; Ndukwe et al., 2004; Tiokeng et al., 2015).

Interestingly, during an ethnopharmacological study conducted among the Nkundo people (Bandundu province, DR Congo), Musuyu Muganza (2006) discovered that some traditional healers use the stem bark of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* for malaria therapy. Based on this finding, the antiplasmodial properties of lyophilized aqueous extracts from these plant parts have been assessed (Muganza et al., 2012; Maloueki et al., 2015). The authors found that *Enantia olivacea* has promising *in vitro* antiplasmodial activity against the chloroquine-resistant *Plasmodium falciparum* K1 strain (IC_{50}=7.77 µg/ml). By contrast, *Garcinia punctata* (IC_{50}=36.56 µg/ml) and *Massularia acuminata* (IC_{50} > 64 µg/ml) were less active against the same K1 strain. Based on all these data, the present study aimed at evaluating the *in vivo* antimalarial efficacy of these three plants either alone or in combination in *Plasmodium berghei*-infected mice.

According to Reagan-Shaw et al. (2007), the doses used for humans are

approximately the twelfth of the doses used for mice. Based on this mouse equivalence of the therapeutic regimens employed for humans, the single dose of 200 mg/kg × 4 days has been chosen as the highest and appropriate dose regimen for testing the antimalarial efficacy of our extracts in mice, in agreement with Rasoanaivo et al. (2004). At this dose regimen, the three plant extracts exhibited different antimalarial efficacy in mice. The highest activity was observed with *Enantia olivacea* followed by *Garcinia punctata* and *Massularia acuminata.*

The efficacy of *Enantia olivacea* and *Garcinia punctata* in both parasite suppression and survival time may be attributable to alkaloids, flavonoids and tannins present in their ethanolic extracts (data not shown). Indeed, many compounds belonging to these phytochemical groups were previously reported to exhibit antimalarial activity (Ambe et al., 2006; Bero et al., 2009; Ngbolua et al., 2011).

These results demonstrate that, at the administered dose, the three extracts were not able to delay the course of an early infection of malaria in mice. In order to overcome incomplete cure, recrudescence and resistance development, the combinatorial drug therapy has been recently introduced for the treatment of parasitic and infectious diseases (Memvanga et al., 2015). In line with this approach, some Congolese traditional practitioners use mixtures of *Enantia olivacea*, *Garcinia punctata* and *Massularia acuminata* extracts as remedies to treat some diseases including malaria. In the current study, the results indicate that the combination effectively potentiates the antimalarial activity of the three plant parts in mice.

Based on these results, some hypotheses can address these observations. First, several phytochemical constituents could re-precipitate and/or be degraded in the gastrointestinal tract, and thus lowering the intestinal absorption and the efficacy of the different extracts. Hence, the development of advanced drug delivery systems such as lipid-based formulations would be useful for improving their oral bioavailability (Li et al., 2011; Memvanga and Préat, 2012; Memvanga et al., 2013). Secondly, increased doses of the combined extract or longer treatment with it could provide total clearance of the *Plasmodium* parasite in the blood and prevent recrudescence since large volumes of decoction of these plants are used in traditional medicine by local population.

The safety/ toxicity profile of these herbal remedies will play a crucial role in ensuring their therapeutic potential. Hence, to check whether the extracts administered orally and transported to the systemic blood circulation could cause damage on the membrane of erythrocytes, the hemolysis test was performed. At concentrations of ≤ 200 µg/ml, the results indicated the suitability of all the extracts for oral administration. Nevertheless, the estimated *in vitro* erythrocyte toxicity may be largely reduced *in vivo* due to possible metabolism of the extracts in the body (Memvanga et al., 2013; 2015). We also examined here the acute oral toxicity of the different extracts administered alone (2,000 mg/kg) or in combination (400-1,200 mg/kg) in mice.

From these observations, the maximum tolerated dose (MTD) and 50 % lethal dose (LD_{50}) of each individual extract is > 2,000 mg/kg. In addition, the MTD and LD_{50} of the combined extract were assumed to be greater than the highest total dose tested, i.e., 1,200 mg of each extract/kg. The MTD is defined as the highest possible dose resulting in no animal deaths, less than 20 % weight loss of the control animals and no particular changes in general signs.

Conclusion

The present study demonstrates for the first time that, in *Plasmodium berghei*-infected mice, *Enantia olivacea* (200 mg/kg, oral) and *Garcinia punctata* (200 mg/kg, oral) exhibited moderate antimalarial activities. By contrast, the ethanolic extract of *Massularia acuminata* (200 mg/kg) displayed only weak antimalarial activity after oral administration. The combination therapy of *Enantia olivacea*, *Garcinia punctata*, *Massularia acuminata* at both 50 and 200 mg/kg of each constituent showed increased protection and survival rate associated with a significant delay of recrudescence compared with each monotherapy. No acute toxicity was observed

in mice treated with a single oral dose of the combined extract (≤ 1,200 mg of each extract/kg). The obtained results can partly justify and support the traditional use of these plant parts for the treatment of uncomplicated malaria in DR Congo. Nevertheless, additional pharmacological (e.g. influence of dose regimen), toxicological (e.g. chronic toxicity, teratogenicity) and chemical (e.g. isolation and identification of active compounds, HPLC fingerprinting etc.) studies would be worthy of consideration.

COMPETING INTERESTS

The authors declare that they have no competing interests

AUTHORS' CONTRIBUTIONS

PKK, DMM and PBM conceived and designed the experiments. PKK, DMM, UM and MMM performed the experiments. PKK and PBM analyzed the data and wrote the manuscript. DMM, JNL, ANBM and PBM provided intellectual input on the paper and reviewed the paper. All authors read and approved the final manuscript. PBM planned and supervised all experiments and manuscript preparation.

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