



Original Paper

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Pharmacological screening of some traditionally-used antimalarial plants from the Democratic Republic of Congo compared to their ecological taxonomic equivalence in Madagascar

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ABSTRACT

Hydro-alcoholic extracts of some plant species growing in two different geographical regions (Democratic Republic of Congo and Madagascar) were evaluated for their inhibitory effects on two malaria parasites strains (*P. falciparum* FcM29 & *P. yoelii subsp nigeriensis*) and cytotoxicity towards leukaemia P-388 cell lines. Results indicate that, the antiplasmodial activity of tested plants varied geographically. Plants growing in continent ecosystem are more active *in vitro* while their ecological equivalence inhabiting island ecosystems are more active *in vivo*. It would be conclude that, the development of phytomedicines from plants of different geographical regions selected by bioguided fractionation would allow the populations to reduce the health care cost. The chemotaxonomic approach has also permitted us to detect moderate antiplasmodial activities in *Neobegua mahafaliensis*, a plant species not previously reported as antimalarial in the traditional medicine knowlegde of Madagascar. The use of a pharmacological property such as the antimalarial activity, in this study, in order to establish genetic filiations between the plants species is an original approach.

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INTRODUCTION

More than 40% of the world's population lives in areas where malaria is endemic and each year 300-400 millions cases of infections are recorded. In Africa, official estimations of annual mortality indicate that 1-3 millions cases of deaths are due to

malaria. Most of the victims are children under 5 years of age (Wery, 1995; Mavakala et al., 2003; Nyamangombe et al., 2006). With fast spreading multidrug resistance to commonly used quinoline-based antimalarial drugs by human malaria parasite *Plasmodium falciparum*, the efficient therapeutic approach

is seriously weakened. It is necessary to search for the novel lead compounds with strong therapeutic activity, low toxicity, low cost and original mode of action (Njomnang and Benoit-Vical, 2007).

The Democratic Republic of Congo (DRC) and Madagascar are reputed for the extraordinary richness of their flora (biodiversity) and boast a wide variety of indigenous species (Debroux et al., 2007; Goodman, 2008). These plant species represent an enormous reservoir of new molecules with potential therapeutic activity which is waiting to be discovered.

In the Democratic Republic of Congo as well as in Madagascar, the majority of people rely on traditional medicine for their health care needs because the costs of conventional drugs are unaffordable (Ngbolua et al., 2011).

The aim of the present research was to evaluate the *in vitro* and *in vivo* antimalarial and cytotoxic activities of some medicinal plants from the Democratic Republic of Congo and their ecological taxonomic equivalence in Madagascar. Such information would be useful in evaluating the effectiveness of Congolese traditional medicine (efficacy and safety) and in the identification of other plant species in a genus which contains a potent antimalarial plant as it is the case for *Neobegua mahafaliensis*, a plant species endemic to Madagascar not previously reported in Malagasy traditional medicine as antimalarial.

Congolese plants studied have been selected as antimalarial plants based on empirical evidence of their clinical use by Congolese traditional healers while plants from Madagascar were selected according to the chemotaxonomic criteria on the assumption that closely related species should be expected to have a greater degree of similarity in the molecular structure of their bio molecules (nucleic acids, proteins, bioactive secondary metabolites) (Minkoff, 1983; Ngbolua et al., 2011). Pharmacological screening comparisons between species originating from different geographic regions

separated by a physical barrier will be useful in assessing the relative biochemical similarities among them. This will serve as a new tool and independent check on evolutionary success of such species. We successfully used this approach in the past for detecting antimalarial activity in *Vernonia cinerea subsp vialis* endemic to Madagascar (Ngbolua et al., 2011) and now we wish to report in this paper the results of a similar pharmacological screening from three Congolese medicinal plants belonging to three different families and their ecotypes growing in Madagascar.

MATERIALS AND METHODS

Plants and extracts

Plants were collected and identified by botanists Clément BOTEFA (*Entandrophragma palustre*) and Jonas ZAMENA (*Catharanthus roseus* and *Senna occidentalis*). Voucher specimens were deposited at EALA botanical garden (Equateur Province, DR Congo) and Herbarium of the Faculty of Sciences (Université de Kinshasa, DR Congo) respectively. Plants from Madagascar were collected during a survey on the ethnomedical use of Congolese antimalarial ecotypes plants, genus or close species conducted among traditional healers in various places in the South of Madagascar during a scientific expedition from July to August 2010. Plants were identified by botanist BENJA of the "Institut Malgache de Recherches Appliquées, IMRA". Voucher specimens have been deposited in the Herbarium of IMRA. Congolese plants were selected based on the number of citations from different *traditional* healers and the wide distribution of their use in DRC as well as in other regions of Africa (Tona et al., 2004). The Malagasy plant ecotypes were selected targeting chemotaxonomic criteria. No antimalarial treatment in Malagasy traditional medicine was recorded by all the contacted traditional healers permitting to test the hypothesis. The dried and powdered leaves (50 g) were repeatedly extracted by cold percolation with

ethanol 90° EtOH (200 ml x 1) for 72 hrs. Chlorophyll from fractions of each plant was removed using activated carbon. Filtrates were mixed and the solvent was evaporated under reduced pressure using a rotary evaporator.

Antimalarial bioassays

Parasites strain and in vitro culture conditions

The asexual erythrocytes' stages of *Plasmodium falciparum* FcM29-Cameroon, a highly chloroquine-resistant strain were grown continuously in stock cultures by a modification of the methods of Trager and Jensen (1976) using glucose-enriched RPMI 1640 medium, supplemented with 10% human serum at 37 °C as previously described (Rafatro et al., 2000).

In vitro antiplasmodial activity

The antiplasmodial activity of the plant extracts was evaluated by an isotopic micro test which determines the inhibition of radio labeled hypoxanthine uptake by malaria parasite as an indicator of growth.

Test extract preparation

Methanol (MeOH, 200 µl) was added to 1 g sample of extracts and further diluted as required in water. The MeOH concentration for tested dilutions was not greater than 1% (Lohombo et al., 2004). Initial concentration of the plant extracts was 50 µg/ml diluted with five-fold dilutions to make five concentrations, the lowest being 0.08 µg/ml. Each test included an untreated control with the solvent and a positive control: chloroquine sulphate (Sigma, France) and ethanolic crude extract of *Cinchona* stem bark.

Isotopic micro test

Two hundred micro litres (200 µl) of total culture medium with the diluted extract (20 µl) and the suspension (180 µl) of *Plasmodium falciparum*-infected human red blood cells in medium (O+ group, 1% haematocrit) with 1% asynchronous parasitaemia was placed into the wells of 96-well micro titre plates. After 18 h incubation of the parasites with the extracts at 37 °C, [³H] hypoxanthine (Amersham, UK) was added to

each well and the incubation was continued for another 24 h at the same conditions.

The mean values for uptake of ³H-hypoxanthine (disintegration per minute, dpm) in control (untreated) and tested parasitized erythrocytes, expressed as the percentage of inhibition, were calculated as previously reported (Ngbolua et al., 2011).

The antimalarial activity of extracts was expressed by the inhibitory concentrations 50% (IC₅₀), representing the concentration of drug that induced 50% parasitaemia decrease compared to control culture. The extract concentration at which the parasite growth (ie [³H] hypoxanthine uptake) is inhibited by 50% (IC₅₀) was calculated by a non-linear regression analysis processed on dose-response curves with the help of Mikro Win Hidex 2000 software. Liquid scintillation counting was operated on CHAMELEON™V multilabel counter plate.

In vivo antiplasmodial activity

Suppressive parasitaemia assay

The *in vivo* antimalarial activity of plant extracts was determined by the classical 4-day suppressive test against *Plasmodium yoelii* subsp *nigeriensis* strain. Briefly, adult male Swiss albino mice weighing 18 to 22 g were inoculated by intravenous (i.v.) route with 10⁷ *Plasmodium yoelii* infected red blood cells. The mice were randomly divided in groups of five per batch, and treated during four consecutive days with daily doses of the extracts, by oral route. Two control groups were used in each experiment, one was treated with ethanolic crude extract of *Cinchona* stem bark (100 mg/kg, orally), the other group was kept untreated. On the 5th day after parasite inoculation, blood smears were prepared from all mice, fixed with methanol, stained with Diff Quick® RAL dyes, then microscopically examined (800 × magnifications).

Counting

Parasitaemia was determined in coded blood smears by counting 2'000 – 6'000 erythrocytes in the case of low parasitaemia (≤1%); or up to 1'000 erythrocytes in the case of higher parasitaemia. The parasitaemia for

each mouse was obtained, and the percentage inhibition of parasitaemia for each dose of extracts was calculated as previously reported (Ngbolua et al., 2011).

The extracts were considered active if parasitaemia was reduced by 33, 63% or more. All extracts were tested at daily doses of 500 mg/kg body weight (Abosi and Raseroka, 2004).

Cytotoxicity assay

***In vitro* cell culture and test protocol**

Cytotoxicity was determined against mouse leukaemia cell line P388. Cells were cultured in RPMI 1640 (Gibco-BRL) supplemented with 10% (v/v) foetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin and 50 mM 2-mercaptoethanol at 37 °C with 5% CO₂.

Briefly, 5×10^3 cells (based on cell growth characteristics) in 180 µl medium were seeded to each of 96 wells in a microtiter plate (3 wells/dose). Various concentrations of plant extract diluted in 20 µl cell medium were added. The cells were incubated at 37 °C, 5% CO₂ and 100% humidity. Cell viability was assessed with the neutral red assay, which is based on the uptake and intracellular accumulation of the supra vital dye.

Neutral red (NR) assay

Following 72 h incubation with test solution, the cells were incubated with neutral red dye to assess cytotoxicity. Viable cells actively transport this dye across their cell membrane, therefore upon subsequent lysis, absorbance can be used as a measure of cell viability. A foil-wrapped 20 mg/ml methanol stock suspension of NR was stored at room temperature. The stock was diluted to a working concentration of 100 µg/ml NR in exposure medium and incubated overnight at 37 °C. Prior to use, this solution was centrifuged to remove fine dye crystals. After a 72 h exposure with the test agents, the medium was removed, 100 µl of NR-containing medium (freshly prepared neutral red solution pre warmed to 37 °C) was added per well, and incubation was continued for 1 h

at 37 °C. The cells were washed three times with PBS. Following draining of the plates, 100 µl of lauryl sulfate solution (1%, Sodium Dodecyl Sulfate, Sigma, Germany) was added to each well and plates were shaken on an orbital plate shaker for 10 min at room temperature to release all of the dye from the cells. Samples were transferred to cuvettes and absorbency was recorded at 540 nm on a microtiter plate spectrophotometer (TitertekTwinreader, Finland). Inhibition of cell proliferation was determined and expressed as per cent of absorbance of NR extracted from control cells (defined as 100%). IC₅₀ values were determined by linear regression method (Rasoanaivo et al., 2004).

Statistical analysis

The results of *in vitro* study are given as Mean ± Standard Deviation obtained from three independent experiments. The results of *in vivo* study were expressed also as Mean ± Standard Deviation and analyzed with Student's t-test for paired data using Origin 6.1 package software. All data were analyzed at a 95% confidence interval ($\alpha=0.05$).

RESULTS

The results of *in vitro* and *in vivo* antimalarial and cytotoxic activities of tested plants are summarized in Table 1.

It is deduced from Table 1 that the antimalarial activity of the tested plant species depends closely on the geographical area of harvest (Effect of climate). Two Congolese plants out of three revealed a moderate *in vitro* antimalarial activity (i.e $10 < IC_{50} < 50$ µg/mL: *C. roseus* IC₅₀ = 41, 7µg/mL; *S. occidentalis* IC₅₀ = 16 µg/mL) against only one Malagasy plant (*N. mahafaliensis* IC₅₀ = 29 µg/mL). While for the *in vivo* antimalarial bioassay, two Malagasy plants out of three possessed very good activity at the oral dose of 500 mg/kg of body weight (i.e %I ≥ 33.00 ± 2.63: *C. roseus* %I=51.00 ± 3.00; *S. occidentalis* %I=69.00 ± 4.00) against a Congolese plant *Catharanthus roseus* (%I=33.00 ± 2.00) (Gessler et al., 1994).

Table 1: *In vitro* and *in vivo* antimalarial and cytotoxic activities of medicinal plants from the Democratic Republic of Congo compared to its ecological taxonomic equivalence in Madagascar.

Family	Medicinal plant (origin)	Used parts	IC ₅₀ (µg/ml)		Therapeutic index	% chemosuppression (<i>P. yoelii</i>)
			Pf FCM 29	P388 Cells		
Apocynaceae	<i>C. roseus</i> (DRC)	Leaves	41.00 ± 6.97	4.00 ± 0.60	0.10	33.00 ± 2.00
	<i>C. roseus</i> (Madagascar)	Leaves	102.00 ± 15.30	64.00 ± 10.88	N/A	51.00 ± 3.00
Milliaceae	<i>E. palustre</i> (DRC)	Stem bark	97.00 ± 13.58	23.00 ± 3.22	N/A	12.00 ± 0.60
	<i>N. mahafaliensis</i> (Madagascar)	Stem bark	29.00 ± 5.22	34.00 ± 5.43	1.20	18.00 ± 0.90
Mimosaceae	<i>S. occidentalis</i> (DRC)	Leaves	16.00 ± 2.56	226.00 ± 42.90	14.60	24.00 ± 1.20
	<i>S. occidentalis</i> (Madagascar)	Leaves	214.00 ± 38.52	43.00 ± 7.74	N/A	69.00 ± 40

Positive control: *Chloroquine* (IC₅₀=265, 48±45,13nM); *Cinchona sp* ethanolic crude extracts 100mg/kg (% Chemosuppression of *P. yoelii*=33, 00±2, 63); *Camptothecin* 5µM (% Inhibition of P388 cell lines = 93, 1± 3, 1); N/A: not applicable; DRC: Democratic Republic of Congo.

DISCUSSION

The results obtained in this research work indicate that the plant species originating from the Democratic Republic of Congo are more active *in vitro* than *in vivo* while those of Madagascar are on the contrary more active *in vivo* than *in vitro*.

These differences in the biological activities could be due to the differences in qualitative and/or quantitative phytochemical content of the extracts. Indeed, it is well-known that genes regulation and in particular the enzymatic contents which catalyze the biochemical pathways of secondary metabolites synthesis in plant are controlled by environmental factors including the climate, the geological nature of the site of harvest and the period of harvest of plants samples (Potchoo et al., 2008, Boudet, 2007, Pieters and Vlietinck, 2005). This geographic variation in antiplasmodial activity would be a result of biochemical adaptations (ecophysiology) that occurred in Malagasy plants in order to make them better suited to

their environment (Minkoff, 1983). So, if one considers that the biosynthesized compounds of the plant let it to adapt to its environmental conditions, the one of south of Madagascar being more arid than that of DRC thus justifying the observed differences.

It should be noted that although the antimalarial effectiveness of *E. palustre* has not demonstrated *in vitro* as well as *in vivo* (no direct effect on parasites), it could be a question of a plant used by traditional healers to alleviate or prevent a wide range of malaria symptoms because of its anti-inflammatory, immunostimulant, antipyretic or vasodilator effects or a plant species which potentiates other plants and thus its effectiveness would depend on associations of the plants (Rasoanaivo et al., 2004).

However, the significant findings of this research is the use of ethno pharmacological approach from the Congolese traditional pharmacopeia related to *E. palustre* to identify a new antimalarial plant *N. mahafaliensis* not previously reported

in Madagascar as antimalarial. This strategy has been successfully used by us in the past to register another Malagasy endemic plant species *Vernonia cinerea* Subsp *Vialis* in the traditional pharmacopeia of Madagascar as a new antimalarial plant (Ngbolua et al., 2011).

It should be also noted that the biological activities of secondary metabolites are due to their chemical structure. Some of these active compounds may be closely found in several genera of a botanical family as the case of *E. palustre* and *N. mahafaliensis* (Meliaceae family). However, the dose of active principle in these plants species depends on the ecological factors which are different in both the Democratic Republic of Congo and Madagascar (Goodman, 2008; Debroux, 2007). In this order of idea, the compound responsible for the antimalarial activity in *N. mahafaliensis* would be masked in *E. palustre*. The presence of such a compound would also make it possible to justify the use of *E. palustre* in Congolese traditional medicine. *Neobegonia mahafaliensis* is a medicinal plant widely used in the South and West parts of Madagascar for the treatment of erectile and libido dysfunction in old men (Wikberg et al., 2008). During our scientific expedition in Madagascar, no antimalarial use was recorded by traditional healers.

The cytotoxicity of all tested plant extracts towards P-338 cell lines was weak ($CI_{50} \geq 23 \mu\text{g/mL}$), this attest that the biological activity of these plant extracts is specific and selective towards chloroquine resistant FCM 29 Cameroon *P. falciparum* strains. However, it should be noted that *S. occidentalis* showed a good therapeutic index. So, more this index is high more the extract is selective and consequently, plant extract is effective and safe. Moreover; all plant extracts tested in this study had no effect on *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and *S. fecalis* indicating also that plant extracts are highly specific to *Plasmodium spp.* This finding corroborates with those reported by other authors who have reported the *in vitro*

antimalarial activity of *Senna occidentalis*. However, the IC_{50} value obtained in this study is greater than that of the result reported by Tona and co-workers (2004) for the same plant. This activity is all the more interesting as it relates to chloroquine resistant strains of malaria parasites and can thus constitute a therapeutic alternative to this drug and its derivatives.

In addition, *Catharanthus roseus* from the Democratic Republic of Congo was found to be cytotoxic against P388 cell lines with an IC_{50} value less than $10 \mu\text{g/mL}$. Its use in Congolese traditional medicine as antimalarial is a poison for the population.

The use of a pharmacological property such as the antimalarial activity, in this study, in order to establish genetic filiation between the plants species is an original approach.

Molecular and phytochemical studies carried out in the families of Ancistrocladaceae and Dioncophylaceae showed that naphthylisoquinoline alkaloids are a specific phytochemical marker of these paleotropical families. It confers to all members the antimalarial properties (Bringmann et al., 2006, 2008; Heubl et al., 2010). Thus, phylogenetic relations between plant species could be established targeting antimalarial activity because biological activity is closely linked to a given phytochemical marker. And the biogenesis of secondary metabolites is genetically controlled (Ngbolua et al., 2011).

Conclusion

The objectives of this study were firstly to validate scientifically the antimalarial effectiveness and safety of *Catharanthus roseus*, *Entandrophragma palustre* and *Senna occidentalis*. The second step was to harvest and evaluate the antimalarial activities of their ecotypes in order to check the effects of spatial isolation on the evolution of the antimalarial properties in such species growing in Madagascar. The results of the present research indicate that the antiplasmodial activity of tested plants varies geographically. Plants growing in continental ecosystems such as DRC are active in an *in*

in vitro bioassay while their ecological equivalences inhabiting island ecosystems like Madagascar are more active in an *in vivo* bioassay.

The development of a traditional remedy derived from plants originating from different geographical regions selected by bioguidance, can potentiate each active ingredient that it contains and thus allow effectiveness comparable with that of the drugs marketed currently and that at lower cost.

To the best of our knowledge, the use of antimalarial properties as a phenotypic marker for understanding the evolutionary biology of African plant species ecotypes growing in Madagascar after many years of spatial isolation and speciation targeting ecological and taxonomical criteria (chemotaxonomy) has not yet been previously reported in the literature before our work. Phytochemical studies on *N. mahafaliensis*, involving chromatographic fractionation are still in progress.

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