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Comparative antimalarial and cytotoxic activities of two *Vernonia* species: *V. amygdalina* from the Democratic Republic of Congo and *V. cinerea subsp vialis* endemic to Madagascar

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ABSTRACT

Vernonia amygdalina Del. is a conventional herbal drug in Congolese traditional medicine and is widely used for the treatment of malaria.The aim of this work is to evaluate its efficacy and safety and the potential antimalarial activity of another species originating from Madagascar. Standard bioassay models based on *in vitro* and *in vivo* systems that enable bioactivity screening of traditionally used medicinal plants were used. In particular, hydro-alcoholic extracts of two Vernonia species growing in two different geographical regions(Congo DR and Madagascar) were evaluated for the inhibitory effects on two malaria parasites strains and cytotoxicity towards leukaemia P-388 cell lines. Results indicate that, *V. amygdalina* possess a very good *in vitro* and *in vivo* activities and a good therapeutic index than *V. cinerea subsp vialis* endemic to Madagascar, thus validate scientifically the efficacy and safety of *Vernonia amygdalina* in the traditional treatment of malaria in Congo DR. Using chemotaxonomic approach, we also detected moderate antiplasmodial activities in *V. cinerea subsp vialis* a plant species not previously reported as antimalarial in the traditional medicine knowlegde of Madagascar. It would be concluded that despite the long spatial isolation of Madagascar and allopatric speciation, Vernonia ecotype as *V. cinerea subsp vialis* has preserved the *antiplasmodial* properties. This approach gives the possibility to select plant species of the same genus from different geographical regions in order to increase the chance of discovering new biologically active plants.

Keywords: Malaria, *Vernonia* species,cytotoxicity, Dem. Rep. of Congo, Madagascar.

INTRODUCTION

The Democratic Republic of Congo (DRC) and Madagascar are reputed for the extraordinary richness of their plant genetic resources. In the DRC, forests cover approximately 62 per cent of the national territory. This is the second largest block of tropical forest in the world. The DRC is

located at the heart of Africa's forest massif and harbors approximately half of the continent's rainforests. Medicinal plants are a key product for the Congolese population, but there is little overall information on the subject. Almost all Congolese populations, both urban and rural, depend on medicinal plants for their health care. This involves hundreds of species, which vary greatly by region. These plants have been found to have therapeutic value for fighting major health problems (Debroux et al., 2007; Goodman, 2008).

In DRC as also in Madagascar, many people rely on traditional medicine for their health care needs because the costs of conventional drugs increase and are becoming unaffordable by many in rural communities. Some of these traditional receipts where found to be useful in an integrated health care system were traditional medicine and orthodox medical systems are combined, as is being practiced in "Institut Malgache des Recherches Appliquées, IMRA" in Madagascar. This practice would be very useful in the Democratic Republic of Congo where tropical disease such as malaria shows the highest prevalence rate in some villages due to the break down of control programs as a consequence of wars and civil strife.

Vernonia amygdalina L., a medicinal plant belonging to the Asteraceae family is used in African traditional medicine for the treatment of various ailments including malaria (Iwalokun, 2008; Njan, 2008; Tabuti, 2008). A clinical trial of the traditional medicine *Vernonia amygdalina* in the treatment of uncomplicated malaria has revealed the efficacy and the safety of this plant (Challand et al., 2009). The plant was reported as anticancer (Gresham et al., 2008; Yedjou et al., 2008; Oyugi et al., 2009), antidiabetic (Gbolade, 2009), antimicrobial (Okigbo and Mmeka, 2008) and in the treatment of measles (Sonibare et al., 2009) and ulcers (Adesanoye and Farombi, 2010). The lipid-lowering effects of *V. amygdalina* were also reported in the literature (Adesanoye et al., 2008). In Africa, *Vernonia amygdalina* is very widely used in human diet as well as a medicine without any reported cases of serious toxicity (Brendler et al., 2010).

The aim of the present study was to obtain more scientific information concerning the efficacy and safety of this plant in the treatment of malaria because recent findings have shown that many plants used in traditional medicine are potentially toxic, mutagenic and carcinogenic (Schimmer et al., 1994; De Sã Ferrira and Ferrão Vargas, 1999). Such information would be useful in evaluating the effectiveness of traditional medicine and in the comparative biological evaluation of others plant species in a genus which contains a potent antimalarial plant as it is the case for *Vernonia cinerea subsp vialis*, a plant species endemic to Madagascar not known as antimalarial plant in Malagasy traditional medicine. Following this trend, this approach will enable to understand how the plants considered as endowed with pharmacological properties on the African continent evolved on a territory insulated by a physical barrier like Madagascar using antimalarial properties *as phenotypic marker of biological evolution in two Vernonia species.*

Moreover, the development of phytotherapeutic agents consists of first conducting a pre-clinical evaluation of the plant extracts used by the community as folk medicine. This evaluation is then followed by biological testing both *in vitro* and *in vivo.* It is only if the extract has an acceptable safety index would it be necessary to conduct detailed pharmacological studies. In this work, *Vernonia amygdalina has been selected* as antimalarial plant based on empirical evidence of it clinical use by traditional healers from DRC.

MATERIALS AND METHODS

Plants and extracts

Vernonia amygdalina Del. leaves collected close to the Ndjili brasseries station (Kinshasa region), were identified by botanist Jonas ZAMENA. A voucher specimen is deposit at the Herbarium of the Faculty of Sciences (Université de Kinshasa, DR Congo). *Vernonia cinerea subsp vialis* leaves were collected during a survey on the ethno medical 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. The plant was identified by botanist BENJA of the "Institut Malgache de Recherches Appliquées, IMRA". A voucher specimen has been deposited in the Herbarium of IMRA. The Congolese plant *Vernonia amygdalina* was selected based on the number of citations from different traditional healers and the wide distribution of the use of the plant in DRC as well as in others regions of Africa (Jisaka et al., 1992). While the Malagasy endemic ecotype Vernonia cinerea subsp vialis was selected targeting chemotaxonomic criteria. *Vernonia cinerea L. subsp. vialis (D.C.) H. Humb*. also named Famonty in Malagasy language is used in Malagasy traditional medicine against the belly-ache. 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. Fractions of each plant were dechlorophylled with activated carbon, filtered, mixed and the solvent was evaporated under reduced pressure using a rotary evaporator.

Antimalarial bioassays

Parasites strain and in vitro culture conditions

The asexual erythrocytic 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 up take by malaria parasite as an indicator of grown.

Test extract preparation

Methanol (MeOH, 200 µL) was added to 1 mg 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: chloroquinesulfate (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, [^{3}H] 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 ^{3}H-hypoxanthine (disintegration per minute, dpm) in control (untreated) and tested parasitized erythrocytes, expressed as the percentage of inhibition, were calculated using the following formula:

$$\text{Inhibition (\%)} = 100 \times \left[\frac{(\text{mean dpm of untreated parasites} - \text{mean dpm of tested parasites})}{\text{mean dpm of untreated parasites}} \right]$$

The antimalarial activity of extracts was expressed by the inhibitory concentrations 50% (IC_{50}), representing the concentration of drug that induced a 50% parasitemia decrease compared to control culture. The extract concentration at which the parasite growth (ie[^{3}H] hypoxanthine uptake) is inhibited by 50% (IC_{50}) 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^{TM}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 10^7 *Plasmodium yoelii* infected red blood cells. The mice were randomly divided in groups of four 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 extract was calculated in relation to the control as the parasitaemia in the control (non-treated) group minus parasitaemia in the drug-treated group, divided by parasitaemia in the control (non-treated) group, expressed as percentages:

$$\text{Inhibition (\%)} = 100 \times \left[\frac{(\text{parasitaemia of control} - \text{parasitaemia of drug})}{\text{parasitaemia of control}} \right]$$

The extracts were considered active if parasitaemia was reduced by 33% 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% CO_2.

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% CO_2 and 100% humidity. Cell viability was assessed with the neutral red assay, which is based on the uptake and accumulation of the supravital 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.

The protocol for the NR assay was as follows. 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%) (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 (α= 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 this table that *Vernonia amygdalina* exhibited a very good *in vitro antiplasmodial* activity (IC_{50} 3, 58±0, 64) because its 50% inhibitory concentration IC_{50} is lower than 10µg/mL according to the criteria of Gessler (1994). *Vernonia cinerea subsp vialis* is moderately active because it's IC_{50} (21, 92±2, 41) is higher than 10 µg/mL and lower than 50 µg/mL (*i.e* $10< IC_{50} < 50$ µg/mL). Comparing these results, one notes that *Vernonia amygdalina* is *6 fold* more active against chloroquine resistant FCM 29 Cameroon *P. falciparum* strains than *Vernonia cinerea subsp vialis*.

The *cytotoxicity* of these two plant extracts towards P-338 cell lines is weak ($CI_{50} \geq 30$ µg/mL), attesting that the biological activity of these two plant extracts is specific and selective towards chloroquine resistant FCM 29 Cameroon *P. falciparum* strains. However, it should be noted that the index of selectivity of *Vernonia amygdalina* is 5 times better than that of *Vernonia cinerea subsp vialis*. So, the more this index is high, the more the extract is selective and consequently, drug is effective and safe. The observed difference in *antiplasmodial* activity between these two plant species was also highlighted *in vivo*. Indeed, at the oral dose of 500 mg/kg of body weight, *Vernonia amygdalina* produced 62, 28% chemosuppression in mice infected with *P.yoelii* against 35, 79% for *Vernonia cinerea subsp vialis*. However, the parasitic chemosuppression power of *Vernonia cinerea subsp vialis* is higher than that of *Cinchona sp*, a medicinal plant whose antimalarial activity is well established and who was used in this study as positive controls. To this end, our assumption was checked and approaches it allowed to enrich the Malagasy pharmacopeia. Moreover, at the evolutionary biology level, these results indicate that despite insulation of Madagascar by a physical barrier since of million years as a result of continental drift and although these two species are specific to each of two ecosystems (continental Africa & Madagascar), these two plant species preserved the common family characters (antimalarial properties) resulting from the Gondwana supercontinent time.

Table 1 : *In vitro* and *in vivo* antimalarial and cytotoxic activities of two African *Vernonia* species.

Botanical name	Origin	IC_{50} (µg/ml)		*Therapeutic Index*	*% Chemosuppression (P. yoelii)*
		P. falciparum	*P 388 cell lines*		
V. amygdalina	Congo, DR	3,58±0,64	30,82±4,62	8,62	61,28±10,21 (500mg/kg)
V. cinerea subsp vialis	Madagascar	21,92±2,41	41,68±5,42	1,9	35,79±7,16 (500mg/kg)

- **Positive control**: ***Chloroquine*** (IC_{50}=265,48±45,13nM); ***Cinchona sp*** alkaloids crude extract 100mg/kg (% Chemosuppression of *P. yoelii*=33,00±2,63); ***Camptothecin*** 5µM (% Inhibition of P388 cell lines = 93,1± 3,1)

DISCUSSION

The prevalence of malaria in Africa, together with the lack of effective vaccine, and the emergence of parasite resistance to conventional drugs makes it necessary to search for antimalarial plant derived new lead compounds through traditional knowledge. To this end, *Vernonia amygdalina* was selected in this work based on empirical evidence of its clinical use by traditional healers from Democratic Republic of Congo DRC. The genus is characterized by the presence of sesquiterpene lactones which possess antiparasitic activities (Jisaka et al., 1992; Chea et al., 2006; Brendler et al., 2010). These therapeutic properties were used in this investigation as phenotypic marker for understanding the biological evolution of vernonia species in Madagascar after many years of spatial isolation and speciation targeting chemotaxonomic criteria. The *in vitro* and *in vivo* antimalarial activities of *Vernonia cinerea subsp vialis* endemic to Madagascar were found to be moderate. This middle activity might be presumably due to differences in number and type of sesquiterpene lactones commonly found in species of *Vernonia*, as result of environmental and genetic variation. Qualitative variation would be due to interspecific variability of the genes which control the synthesis of the secondary metabolites (Boudet, 2007) while the quantitative variability would reflect environmental factors (chemical races). There are several internal and external factors affecting the quantity and quality of secondary metabolites in a plant species. These include the genetic (varietal and regional) diversity as well as many environmental variables, ie growing conditions such as light intensity, humidity, temperature or others stress factors (Pieters and Vlietinck, 2005). Chromatographic fractionation may improve the *in vitro* and *in vivo* activities of *Vernonia cinerea subsp vialis*, by concentrating the biologically active compounds in some fractions. The study could also confirm the claim in Congolese traditional medicine that the plant *V. amygdalina* has therapeutic values in human malaria as previously reported by others researchers (Abosi and Raseroka, 2004; Iwalokun, 2008; Njan, 2008; Tabuti, 2008; Challand et al., 2009; Brendler et al., 2010). This activity is the most interesting, as it relates to chloroquine resistant strains of malaria parasites and can thus constitute a therapeutic alternative to this drug and its

derivatives. The good therapeutic index observed testifies a good selectivity toward *Plasmodium spp.* However, the IC_{50} value of *Vernonia amygdalina* (IC_{50} < 3 µg/ml) obtained in this study is less than that of the result obtained by Tona and co-workers (5< IC_{50} < 10 µg/ml) for the same plant (Tona et al., 2004).

Moreover, all plant extracts tested in this study had no effect on bacteria such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, S. fecalis* demonstrating that these plants are highly specific to *Plasmodium spp.* The use of medicinal plants is a simple, inexpensive and appropriate strategy for disease control in developing countries such as DRC. There is, thus, the need to initiate further in-depth investigation in order to isolate and characterize the biologically active compounds.

Ethno pharmacologists are based on ethno botanical surveys for their research but they have some difficulties as traditional healers are not easy going with their receipts which are hided by them (Krief, 2004). For this reason, our approach is an alternative which allows us to avoid this difficulty.

Conclusion

The objectives of this study were firstly to validate scientifically the therapeutic efficacy of *Vernonia amygdalina.* The second step was to harvest and evaluate the antimalarial activities of its ecotype *Vernonia cinerea subsp vialis* in order to check the effects of spatial isolation on the evolution of the antimalarial properties in Vernonia genus growing in Madagascar. The results of the present study indicate that the two plants species are biologically active.

The ability of the extracts of the two Vernonia species to inhibit the malaria parasites growth in this study may represent a rational explanation for the use of *Vernonia cinerea subsp vialis* as new source of biologically active compound in the treatment of malaria in Madagascar and validates the traditional phyto-therapeutic claims of *Vernonia amygdalia* as previously reported elsewere.

To the best of our knowledge, a comparative antimalarial screening of plants from African continent and Madagascar has not yet been previously reported in the literature. Phytochemical studies on these species, involving chromatographic fractionation are still in progress.

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