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In vitro and *in vivo* anti-malarial and cytotoxic activities of ethanolic extracts of *Annona senegalensis* Pers (Annonaceae) from Democratic Republic of the Congo

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ABSTRACT

The objective of this study was to validate scientifically the antimalarial effectiveness and safety of *A. senegalensis.* Ethanolic crude extracts of *Annona senegalensis* growing in Democratic Republic of the Congo were evaluated for the 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 activities of tested plant extracts are moderate *in vitro* and weak *in vivo*. While the leaves crude extract displayed toxic effect towards P-388 cells. So, the wide use of this medicinal plant in Congolese Folk Medicine as antimalarial herbal could constitutes a great risk of population poisoning.

Keywords: Malaria, Traditional Medicine, cytotoxicity, Democratic Republic of the Congo.

INTRODUCTION

Malaria is an infectious disease caused by a protozoan of the phylum: Apicomplexa, class: Sporozoa, subclass: Coccidia, suborder: Haemosporina, family: Plasmodidae, genus Plasmodium and transmitted by the female mosquito of the genus Anopheles when it feeds by sucking human blood and whose life cycle alternates between man and mosquito (Saganuwan & Azubuike, 2014). Recent reports indicate that more than 40% of the world's population lives in regions where malaria is endemic and each year about 300-400 million cases of malaria infections are recorded. In Africa, official estimations of annual mortality indicate that 1-3 million cases of deaths are due to malaria. Most of the victims are children under the age of 5 years (Wery, 1995; Mavakala *et al.*, 2003, Ngbolua *et al.*, 2011a, b). With fast spreading multidrug resistance to commonly used quinoline-based antimalarial drugs by human malaria parasite *Plasmodium falciparum*, the efficient therapeutic approach is seriously weaken. It is therefore necessary to search for the novel plant based lead compounds with strong therapeutic activity, low toxicity, low cost and original mode of action (Fatiany et *al.*, 2013).

The Democratic Republic of the Congo (DRC) is reputed for the extraordinary richness of it mega-biodiversity and boast a wide variety of plant species of ethno-medicinal relevance (Ngbolua *et al.*, 2015; Ngbolua *et al.*, 2014a-j; Ngbolua *et al.*, 2013). These plant species represent an enormous reservoir of secondary metabolites with biopharmaceutical potential for modern industries. In DRC, the majority of people rely on folk medicine for their health care needs because the costs of conventional drugs are unaffordable and Traditional Healers from this country have long used plants to prevent or cure infections (Ngbolua *et al.*, 2011a, b).

The present work was undertaken with the aim of evaluating the *in vitro* and *in vivo* antimalarial and cytotoxic activities of *Annona senegalensis* leaves (figure 1) from DRC. Such information would be useful in evaluating the effectiveness of Congolese traditional medicine (efficacy and safety).

This plant species has been selected based on empirical evidence of it clinical use by Congolese traditional healers.

Figure 1: The leaves of Annona senegalensis Pers (Annonaceae)

MATERIALS AND METHODS

Plant collection and preparation of extracts

Plant was collected and identified by botanist Jonas ZAMENA. Voucher specimen is deposit at Herbarium of the Faculty of Science (Université de Kinshasa, DRC). Plant species 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 (Ajaiyeoba et *al*., 2006). 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 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 method of Trager and Jensen using glucose-enriched RPMI 1640 medium, supplemented with 10% human serum at 37 °C as previously described (Fatiany et *al*., 2013; Ngbolua et *al*., 2011a, b).

***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 gm sample of extract and further diluted as required in water. The MeOH concentration for tested dilutions was not greater than 1% (Fatiany et *al*., 2013; Ngbolua *et al*., 2011a, b**)**. 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, [^{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 as previously reported (Ngbolua *et al*., 2011a, b).

The antimalarial activity of extracts was expressed by the inhibitory concentrations 50% (IC_{50}), representing the concentration of drug that induced 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 (Fatiany et *al*., 2013; Ngbolua *et al*., 2011a, b**)**.

***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 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*., 2011a, b).

The extracts were considered active if parasitaemia was reduced by 33, 00±2, 63% or more. Extract was tested at daily dose of 500 mg/kg body weight.

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 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 extract, 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%).

IC_{50} values were determined by linear regression method (Fatiany et *al*., 2014; Fatiany et *al*., 2013; Ngbolua *et al*., 2011a, b).

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 AND DISCUSSION

Table 1: In vitro and in vivo anti-malarial and cytotoxic activities of Annona senegalensis Pers

Family	Medicinal plant (Country of origin)	Used part	IC_{50} (µg/ml)		Therapeutic index	% chemo-suppression (*P. yoelii)*
			P_f FCM 29	P388 Cells		
Annonaceae	*A. Senegalensis* Pers (Democratic Republic of the Congo)	Leaves	32,52±6,97	8,74±0,60	0,27	16,93±2,00

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

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

It is deduced from the table 1 that the antimalarial activity of the tested plant extract moderate *in vitro* (i.e 10< IC_{50} < 50 µg/mL: *A. senegalensis* IC_{50} = 32, 52±6, 97 µg/mL) and weak in vivo (i.e %I < 33, 00±2: %I=16, 93±2, 00). The ethanolic crude extract from the leaves of *A. senegalensis* displayed also cytotoxic effect towards P-388 cells (IC_{50} < 10 µg/mL, therapeutic index= 0, 27). These results are not consistent with previously reported research work. Indeed, Ajaiyeoba et *al*. (2006) reported that *A. senegalensis* harvested in Nigeria had intrinsic antimalarial property that was dose- dependent. They found that, at dose of 100 mg/kg body weight of mice, methanolic extract produced significant chemosuppression of parasitemia (> 57%) when administered orally. It had the highest activity at 800 mg/kg weight of mice (91.1%). Their extract exhibited low cytotoxicity against A2780 ovarian cancer cells (with an IC_{50} of 28, 8 µg/ml).

In the present study, the antimalarial effectiveness of *A. senegalensis* from DRC has not formerly demonstrated *in vivo*, it could be a question of a plant which used by traditional healers to alleviate or prevent a wide range of malaria symptoms because of its anti-inflammatory, immunostimulant, antipyretic or vasorelaxant effects or a plant species which potentiates other plants and thus its effectiveness would depend on associations of the plants (Rasoanaivo *et al*., 2004). Although, *A. senegalensis* was found to be cytotoxic against P388 cell lines with an IC_{50} value less than 10µg/ml (weak therapeutic index). The observed cytotoxic effect of the leaves could be due to presence of aporphine alkaloid. Indeed, You and coworkers (1995) reported that (-)-roemerine, isolated from the leaves of *A. senegalensis*, was found to enhance the cytotoxic response mediated by vinblastine in multidrug-resistant KB-V1 cells. So, the wide use of this medicinal plant in Congolese Folk Medicine as antimalarial could constitutes a great risk of population poisoning. The geographic variation in the bioactivity of *A. senegalensis* (Democratic Republic of the Congo and Nigeria) could be due to the differences in qualitative and/or quantitative phytochemical content of the plant species. Indeed, it is well-known that the regulation of genes governing the biochemical pathways of secondary metabolites synthesis in plant are controlled by the environmental factors including the climate, the geological nature of the site of harvest and the period of the harvest of the plants samples (*Potchoo et al*., 2008, Boudet, 2007, Pieters and Vlietinck, 2005).

CONCLUSION

The objective of this study was to validate scientifically the antimalarial effectiveness and safety of *A. senegalensis*. The results of the present study indicate that the antiplasmodial activity of tested plant species is moderate. However this plant species displayed cytotoxic effects against P388 cells. So, the wide use of this medicinal plant in Congolese Folk Medicine as antimalarial herbal could constitutes a great risk of population poisoning.

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