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ORIGINAL ARTICLE

Bioactivity validation of *Phyllanthus amarus* using cell lines as model systems

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ABSTRACT

Medicinal plants constitute an important component of flora and are widely distributed in the Democratic Republic of the Congo (DRC). The aim of the present study was to validate scientifically both the anti-malarial effectiveness and safety of Phyllanthus amarus a medicinal plant traditionally used to treat malaria in DRC using standardized model systems. Ethanolic crude extracts of P. amarus were evaluated for the inhibitory effects on two malaria parasites strains (P. falciparum FcM29 & P. yoelii subsp nigeriensis) while cytotoxicity of the plant extract was carried out against leukaemia P-388 cell lines. Results indicate that, P. amarus displayed good activity both in vitro and in vivo. While the crude extract displayed toxic effect towards P-388 cells. So, the wide use of this medicinal plant in Congolese Folk Medicine as anti-malarial herbal could constitutes a great risk of population poisoning. From this study, it can be predicted that P. amarus possess secondary metabolites with considerable cytotoxic potency and could lead to the discovery of useful anti-cancer drugs of plant origin.

Keywords: *Malaria, Phyllanthus amarus, Traditional Medicine, cytotoxicity, Democratic Republic of the Congo*

Introduction

Phyllanthus amarus Schum. & Thonn. is a small herb belonging to the Phyllanthaceae family. This plant is a branching annual glabrous herb which is 30-60 cm high and has slender, leaf-bearing branchlets, distichous leaves which are subsessile, elliptic-oblong, obtuse, rounded base. Flowers are yellowish, whitish or greenish, auxillary, males' flowers in groups of 1-3 whereas females are solitary. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back.[1] The plant species is well known for its medicinal properties and widely used worldwide. In Indian Ayurvedic system of medicine as well as in African Traditional Medicine, the plant is used in various ailments such as stomach, genitourinary system, liver, and kidney and spleen problems. It is bitter, astringent, stomachic, diuretic, febrifuge and antiseptic. The whole plant is used in gonorrhea, menorrhagia and other genital affections. It is useful in gastropathy, diarrhoea, dysentery, ophthalmopathy, scabies, ulcers, wounds and intermittent fevers.[2] In Democratic Republic of the Congo (DRC), the plant is used in the treatment of malaria[3] which is an infectious disease caused by a protozoan of the phylum: Apicomplexa, class: Sporozoa, subclass: Coccidia, suborder: Haemosporina, family: Plasmodidae, genus Plasmodium.[4]

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.[5, 6] With fast spreading multidrug resistance to commonly used quinoline-based antimalarial drug like chloroquine by human malaria parasite *Plasmodium falciparum*, the efficient therapeutic approach is seriously weaken. It is therefore necessary to screen medicinal plants traditionally used to treat malaria by local communities for their therapeutic properties and cytotoxicity.[3, 7-11]

DRC is a big country located in the heart of Africa hosting a wide variety of plant species of ethno-medical relevance.[11, 12-22] These plant species represent an enormous reservoir of secondary metabolites with biopharmaceutical potential for modern industries.

The present work was undertaken with the aim of evaluating the *in vitro* and *in vivo* antimalarial and cytotoxic activities of Phyllanthus amarus from DRC. Such information would be useful in evaluating the effectiveness (efficacy and safety) of such herbal medicine by providing baseline information for large scale exploitation.

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Materials and Methods

Plant identification and preparation of extracts

Plant was identified by Professor Félicien Lukoki Luyeye. Voucher specimen is deposit at Herbarium of the Biology department, Faculty of Science (Université de Kinshasa, DRC). This plant species were selected based on empirical evidence of it clinical use by traditional healers as reported in the literature.[23] 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.[3,7-9,11]

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%. 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

In vitro 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. 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 3H-hypoxanthine (disintegration per minute, dpm) in control (untreated) and tested parasitized erythrocytes, expressed as the percentage of inhibition, were calculated as previously reported.[7,8] 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 CHAMELEONTMV multilabel counter plate.[7-9,11]

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 (iv) 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 2000–6000 erythrocytes in the case of low parasitaemia (≤1%); or up to 1000 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.[3, 7, 8, 11]

The extracts were considered active if parasitaemia was reduced by 3300±2, 63% or

more. Extract was tested at daily dose of 500 mg/kg body weight.

Cytotoxicity assay

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 × 103 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.

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.[3, 7-11]

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

The results of the bioactivity of Phyllanthus amarus extract on the selected model systems are summarized in Table 1.

It is deduced from the table 1 that the *Phyllanthus amarus* displayed a good anti-malarial activity both *in vitro* and *in vivo* (i.e IC_{50} < 10 µg/mL: P. amarus IC_{50} = 5.742±0.416 µg/mL and %I > 33.00±2.630: %I= 34.605±3.501). The ethanolic crude extract from this herbal medicine displayed also cytotoxic effect towards P-388 cells (IC_{50} < 10 µg/mL) with a therapeutic index of 1.006. These results are consistent with previously reported data. Indeed, a recent investigation of the antiplasmodial activity of the extracts of *Phyllanthus amarus* from Nigeria on *Plasmodium yoelii* (a resistant malaria parasite strain used in animal studies) infection in mice revealed a dose-dependent chemotherapeutic activity (with the chemo-suppression rate of 79% at 1600 mg/kg/day).[24] While using Brine shrimp lethality bioassay, it was reported that the whole plant of *P. amarus* from Bangladesh exhibited potent cytotoxicity with a LC50 value of 20.16 µg/mL.[25]

The present study validated the antimalarial effectiveness of *P. amarus* from DRC. Published research works revealed that this herbal medicine have numerous phytocompounds such as alkaloids, flavonoids, tannins, lignins, polyphenolic compounds and tetracyclic triterpenoids. Among phyto-constituents isolated from this plant are Geraniin, corilagin, 1,6-digalloylglucopyranoside rutin, quercetin-3-O-glucopyranoside, Amarulone, Phyllanthusiin D & Amariin (tannins); Niranthin, Nirtetralin, Phyltetralin, Hypophyllanthin, Phyllanthin, demethylenedioxy-niranthin, 5- demethoxy-niranthin, Isolintetralin (lignans); Isobubbialine and Epibubbialine (alkaloids), 2Z, 6Z, 10Z, 14E 18E, 22E-farnesil farnesol (triterpens).

Table 1. Anti-malarial and cytotoxic activities of *Phyllanthus amarus*

Family	Medicinal plant (Country of origin)	Part Used in the present study	IC_{50} (µg/mL)		Therapeutic index	% chemosuppression (*P. yoelii*)
			P_f FCM 29	P388 Cells		
Phyllanthaceae	*P. amarus* Schumach. & Thonn. (Democratic Republic of the Congo)	Whole plant	5.742±0.416	5.776±0,260	1.006	34.605±3.501

(Legend: Positive control: Chloroquine (IC50=265.480±45.130 nM); Cinchona sp ethanolic crude extract 100 mg/kg (%Chemosuppression of P. yoelii=33.000±2.630); Camptothecin 5 µM (% Inhibition of P388 cell lines = 93.100± 3.100).)

Thus justifying it large spectrum of pharmacological activities (including antiviral, antibacterial, antiplasmodial, anti-inflammatory, anti-malarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, hepatoprotective, nephroprotective and diurectic properties) and it use in folk medicine.[1,2] Although, in the present study, we have demonstrated that *P. amarus* is cytotoxic against P388 cell lines with an IC_{50} value less than 10 µg/mL. Hence, the wide use of this medicinal plant in Congolese Folk Medicine as anti-malarial could constitute a great risk of population poisoning.

Conclusions

The goal of this study was to validate scientifically the antimalarial effectiveness and safety of *P. amarus*. The results indicate that this herbal medicine has good in vitro and in vivo antiplasmodial activities. However the 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. From this study, it can be predicted that *P. amarus* possess secondary metabolites with interesting cytotoxic potency and could lead to the discovery of useful anti-cancer drugs of plant origin

Conflict of Interest

None

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