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# Haem Polymerization Inhibitory Activity and Cytotoxicity of Six Medicinal Plants Used in Cameroon for the Management of Malaria

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## ABSTRACT

Malaria was successfully treated with both natural and synthetic products. However, recent progress in battling malaria has stalled due to drug resistance. Therefore, the search of novel antimalarials capable of reversing or evading resistance is much needed and this could be achieved through ethnomedicinal approaches. Six medicinal plants were screened for their antimalarial activity using the β-hematin inhibition (BHI) assay and their effect on the proliferation of three cancer cell lines (A549, MCF7 and PC3) was assessed by the MTT assay. Amongst the twenty-seven extracts screened, *Pseudospondias microcarpa* bark showed significant BHI activities with $IC_{50}$ values of 2.5 ± 0.1 and 4.0 ± 0.2 μg/mL for DCM and MeOH extracts, respectively, while having no cytotoxic effect on A549, MCF7 and PC3. The current results support the ethnopharmacological use of *P. microcarpa* in the treatment of malaria, and it could constitute a useful source of potent antimalarial compounds.

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## INTRODUCTION

Malaria caused by drug resistant *Plasmodium* can prove to be a fatal infection despite both prevention measures (e.g. bed nets) and drug treatment measures in endemic areas[1]. In 2019, there were an estimated 229 million cases claiming 409,000 lives compared to 228 million and 405,000 cases and deaths, respectively, in 2018[2]. Malaria is established in 91 countries and is ubiquitous in sub-Saharan Africa, where around 90% of global deaths have been documented, especially for the under-fives[3]. Global efforts to control and eliminate malaria have been further threatened since the emergence of drug resistance. However, this is not a new phenomenon; resistance to quinine was found in 1910 and to chloroquine in the 1970s[4,5]. One of the most effective recent introductions, artemisinin (and its semi-synthetic derivatives) have their efficacy also diminishing in the Greater Mankong Subregion and the zone of resistance is propagating through economic migration[6,7]. Among existing antimalarial drugs, resistance to quinine has been shown to develop slowly compared to other antimalarials. Therefore, quinine is still in use today and remains the drug of last resort for the treatment of multidrug resistant malaria infection[8,9]. Therefore, there is a continuing search for compounds that may be worthy of clinical development to fight the resistance phenomenon[10,11].

Quinoline antimalarials such as amodiaquine, chloroquine and quinine were found to act at the erythrocytic stage of the malaria parasite by inhibiting the polymerisation of haem to haemozoin[12]. The formation of haemozoin crystals, a critical process in malarial parasite detoxification has been identified as a suitable target for antimalarial drugs development[13]. Specifically, the parasite catabolises haemoglobin both for anabolic parasite replication to make room and for growth. However, in doing so it has the potential to release haem which can accumulates in its vacuoles up to toxic levels thereby killing the parasite[14,15].

In the present study, we evaluated the haem polymerization inhibitory activities of the crude extracts of six medicinal plants from the Cameroonian flora, in a cell free medium, as an indicator of their antimalarial potential. These include *Croton oligandrus* Pierre ex. Hutch, *Entandrophragma congoënse* (DeWild) A. chev., *Pseudospondias microcarpa* (A. Rich.) Engl., *Ruspolia hypocrateriformis* (Vatair feilding ephl) Milne-Redh, *Zanthoxylum lepreurii* Guill. & Perr. and *Zanthoxylum zanthoxyloides* (Lam.) Zepern. & Timler. They are used in

Cameroonian traditional medicine for the treatment of different ailments including malaria and malaria symptoms including anaemia and fever (Table 1)[16-22]. The cytotoxic effects of the plants were also evaluated against three cancer cell lines.

**Figure 1.** Selected medicinal plants studied: Croton oligandrus Pierre ex Hutch leaves a) and stem bark b); *Entandrophragma congoënse* A. Chev. stem bark c); *Ruspolia hypocrateriformis* Vahl Milne-Redh leaves d); *Pseudospondias microcarpa* (A. Rich.) Engl. stem bark e), leaves f) and fruits g); *Zanthoxylum lepreurii* Guill. and Perr. fruits h); *Zanthoxylum zanthoxyloides* (Lam.) Zepern. and Timler fruits i).

## METHODOLOGY

### Plant materials

Materials for extraction were collected from their natural habitat in three sites namely: S1) Mount Eloundem (3⁰49′1.794″N 11⁰25′59.412″E); S2) Melen (3⁰51′51.559″N 11⁰30′8.748″E) in Yaoundé, Centre-Cameroon and S3) Tsenfem (5⁰27′12.052″N 10⁰3′9.021″E) in Dschang, West-Cameroon. All collected plant parts (Figure 1) were identified and authenticated at the Cameroon National Herbarium Yaoundé by Mr Nana where their voucher specimens have also been deposited (Table 1).

### Preparation of crudes extracts

Air-dried and ground plant material (200 g for each plant part collected) were extracted, successively, with *n*-hexane (HEX), dichloromethane (DCM) and methanol (MeOH), 800 mL using a Soxhlet extractor. Ten cycles were completed for each successive solvent extraction. The extracts were filtered and evaporated to dryness using a rotary evaporator at a temperature not exceeding 40 °C and subsequently kept at 4°C until required[22].

### Evaluation of the antimalarial activity using *β*-hematin assay

The cell-free method described by Afshar et al[23] with some modifications, was used. Different concentrations of the extracts (10 μL, 0-250 μg/mL) prepared in DMSO were incubated with 100 μL of 3 mM of hematin, 10 μL of 10 mM oleic acid and 10 μL of 1 M HCl. Sodium acetate buffer 500 mM, pH 5 was used to adjust the volume to 1000 μL. All the solutions were pre-warmed at 40°C before initial mixing. The samples were incubated overnight at 37 °C with regular shaking. After incubation, samples were centrifuged (14,000 x g, 10 min, at 21 °C) to precipitate haemozoin pellets. This was then washed four times with sonication (30 min, at 21 °C; FS100 bath sonicator; Decon Ultrasonics Ltd.) using a solution of 2.5% (w/v) SDS in phosphate-buffered saline followed by a final wash in 0.1 M sodium bicarbonate, pH 9.0, until the supernatant was clear. After the final wash, the supernatant was removed, and the pellets were re-suspended in 0.5 mL of 0.1 M NaOH and transferred to a 96 well plate before determining the haemozoin content by measuring the absorbance at the Soret band (405 nm). The results were recorded as % inhibition (I%) of haem polymerization/crystallization using the following formula: I% = [(AB–AA)/AB] x 100, where AB: absorbance of blank (medium without sample containing 10 μL DMSO instead); AA: absorbance of test samples. Chloroquine diphosphate (prepared in distilled water) and medium without any extract were used as positive and negative controls, respectively.

### *In vitro* cytotoxic assay

The cytotoxicity of the crude extracts against A549 (adenocarcinoma human alveolar basal epithelial cell line), MCF7 (human breast adenocarcinoma cell line) and PC3 (human prostate cancer cell line) was evaluated using the MTT assay.[24] The cells were grown in RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% foetal bovine serum (FBS) and cultured at 37°C, 5% $CO_2$ and 95% humidity. For conducting the assay, the cells were seeded into 96 well plates ($1.2\times10^4$/well) and incubated for 24 h. Cells were then treated with crude extract (0-250 μg/mL) for 24 h and the cell viability measured. Stock solutions (500 mg/mL) of crude extracts were prepared in DMSO and dilutions were made in cell culture media, with the final concentration of DMSO being below 0.1%. The resultant formazan crystals were dissolved in DMSO and optical density was read at 570 nm using a ClarioStar microplate reader (BMG Labtech, UK). The cytotoxicity was determined using the percentage absorbance compared to control cells [(absorbance of treated cells/absorbance of untreated cells) × 100].

### Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± SEM (standard error of mean). $IC_{50}$ values were calculated using the software Graphad Prism 7.02 (GraphPad Prism Software Inc., USA). Differences in means were estimated by means of repetitive measures followed by Mann Whitney test. Differences between means were regarded significant at $p<0.05$.

## RESULTS and DISCUSSION

Plants are the almost exclusive source of healthcare for 80% of the world's population and particularly in the developing countries[25]. The ethnopharmacological properties of plants have been used as a primary foundation for drug discovery[26,27]. Natural products continue to provide a useful source of new drugs as evident from a four-decade review[28]. Fabricant & Farnsworth[29] demonstrated that 80% of the 94 medicinal plants (from which 122 compounds have been isolated and used as drugs) possess an ethnomedicine use indistinguishable (or related) to the current use of the active plant principle. The importance of ethnopharmacological studies involving medicinal plants remains important in drug discovery and the pharma sector has shown renewed interest especially for uncovering new prototypes to tackle drug resistance[30,31].

In the current investigation, the six plants from five families screened were selected on the basis of their use in traditional medicine in Cameroon for the treatment of malaria and associated symptoms, especially anaemia and fever.

A total of twenty-seven extracts were obtained from the different parts of the six medicinal plants collected. The extracting solvents used successively extracted the constituents of the plant gradually according to their affinity and increasing dielectric constant with each solvent[32]. The highest extraction yield was recorded for *Z. zanthoxyloides* fruits (13.7 %), while *P. microcarpa* stem bark provided the lowest extraction percentage yield of 3.3%(Table 1). Screening of the extracts for their haem polymerization inhibitory activity revealed three extracts that exhibited promising antimalarial activity with $IC_{50} \leq 30$ μg/mL. The results are shown in Table 2. The DCM and the MeOH stem bark extracts of *P. microcarpa* showed significant activity with $IC_{50}$ values of 2.5 ± 1.5 and 4.0 ± 1.7 μg/mL, respectively. The fruit extracts of the same plant were inactive, whereas only the MeOH extract of the leaves showed better activity with $IC_{50}$ of 13.0 ± 9.0 μg/mL. Interestingly, similar results were previously reported for the ethanol extracts of *P. microcarpa* stem bark and leaves $IC_{50}$ 1.13± 0.16 and 26 ± 10 μg/mL, respectively, when tested *in vitro* against the multidrug resistant *Plasmodium falciparum* K1 and chloroquine-resistant FCM29

Cameroonian strains, respectively[33,34]. This suggests the haemozoin assay is a suitable assay to probe the antimalarial activity.

The twenty-seven extracts were also evaluated for *in vitro* cytotoxicity against A549, MCF7 and PC3. Doxorubicin was used as the reference drug. Most of the screened extracts exhibited low or no toxicity at the highest concentration tested against the assayed cell lines with $IC_{50}$ values ≥50 μg/mL (Table 3). *E. congoënse* stem bark extracts were found to be the most cytotoxic against all the cell lines tested. A moderate cytotoxicity was observed for its *n*-hexane extracts against A549 and MCF7, and DCM extract against PC3 with $IC_{50}$ = 28.1 ± 6.3, 40.3 ± 5.2 and 32.2± 6.1 μg/mL, respectively, while significant toxicity against MCF7 ($IC_{50}$ = 21.6 ± 0.9 μg/mL) was exhibited by the DCM extract. Extracts of *P. microcarpa,* which demonstrated significant haemozoin inhibitory activity, were found to have no cytotoxic effect on the tested cell lines ($IC_{50}$ values >250 μg/mL). This suggests that extracts that inhibits haemozoin crystallisation may share similar targets and/ or mechanisms of action as acridines

**Table 1.** Medicinal plants studied, place of collection, Voucher number, ethnomedical uses and yield of extraction

| Plant name (Family) | Voucher Nº | Place | Ethnomedical uses | Chemical contents | Plant Part | Weight of extract (g) | | | Yield (%w/w) |
|---|---|---|---|---|---|---|---|---|---|
| **Solvent** | | | | | | **HEX** | **DCM** | **MeOH** | |
| C. *oligandrus* (Euphorbiaceae) | 6687/SFR | S1 | Anaemia, cancer, pneumonia, splenomegaly | Diterpenes | Leaves | 4.91 | 5.7 | 7.07 | 8.8 |
| | | | | | Stem Bark | 2.24 | 1.27 | 4.48 | 4.0 |
| E. *congoense* (Meliaceae) | 43234/SFR | S1 | Gastric ulcer and malaria | Limonoids, steroids, tirucallane triterpenes | Stem Bark | 4.82 | 2.38 | 11.95 | 9.6 |
| P. *microcarpa* (Anacardiaceae) | 41437/SFR | S1 | Anaemia, malaria, helminthiasis | Flavonoids | Fruits | 3.14 | 0.73 | 5.43 | 4.6 |
| | | | | | Leaves | 6.13 | 1.24 | 5.72 | 6.5 |
| | | | | | Stem Bark | 1.6 | 0.8 | 4.27 | 3.3 |
| R. *hypocrateriformis* (Acanthaceae) | 37822/SFR | S2 | Anaemia, diarrhoea, | Flavonoids | Leaves | 6.05 | 4.54 | 18.58 | 14.6 |
| Z. *lepreurii* (Rutaceae) | 106669/ SFR | S3 | Anaemia, malaria | Benzophenantrine, acridone and aporphine alkaloids | Fruits | 11.97 | 7.72 | 19.87 | 19.8 |
| Z. *zanthoxyloides* (Rutaceae) | 21793/SFR | S3 | Anaemia, cancer, fungi infection and malaria | Acridone alkaloids, coumarins, amides, lignans | Fruits | 34.05 | 3.38 | 10.43 | 23.9 |

S1: Mount Eloundem; S2: Melen; S3: Tsenfem; HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol. Yield of extraction is calculated as (the sum extracts weight for a given plant) x 100/ weight of powder (e.g. mepacrine) and 4-aminoquinlines (e.g. chloroquine, amodiaquine) which continue to play a role in the search for drugs that can evade parasite resistance.[4] The inhibition of haemozoin formation in parasites continues to be an attractive target for the development of new antimalarial drugs from medicinal plants[35]. This is due in part, to the idea that the formation of haemozoin in the parasite vacuole is essential for its survival[36]. Consequently, screening plant extracts that can inhibit haemozoin formation can allow rational mechanism-based discovery, for screening ethnomedical plant prototypes.

Our study provides some evidence on the haemozoin inhibitory-antimalarial mode of action as well as safety *in vitro* of *P. microcarpa*, thereby supporting its traditional use for the treatment of malaria. Notably, given that Adongo et al[37] showed that an ethanolic extract of *P. microcarpa* dosed to rats (*per oral*) proved safe. In the light of the afore mentioned results, we therefore, plan to isolate the active component and measure the antimalarial activity of the plant *in vivo* using animal models infected with strains of *Plasmodium* to determine its suitability for further development.

**Table 2.** Inhibition of β-hematin formation assay ($IC_{50}$) of the different extracts

| **Plants** | **$IC_{50}$ (µg/mL)** | | |
|---|---|---|---|
| **Solvents** | **HEX** | **DCM** | **MeOH** |
| *C. oligandrus* L (COL) | > 250 | > 250 | > 250 |
| *C. oligandrus* SB (COBSB) | 180.0 ± 6.0 | 164.8 ± 53.0 | > 250 |
| *E. congoënse* SB (ECSB) | > 250 | > 250 | > 250 |
| *P. microcarpa* F (PMF) | > 250 | > 250 | > 250 |
| *P. microcarpa* L (PML) | > 250 | > 250 | 13.0 ± 9.0 $^{\beta}$ |
| *P. microcarpa* SB (PMSB) | 73.9 ± 25.8* | 2.5 ± 1.5 $^{\beta}$ | 4.0 ± 1.7 $^{\beta}$ |
| *R. hypocrateriformis* L (RHL) | 206.7 ± 52.$^{0}$ | > 250 | 170.3 ± 77.9* |
| *Z. leprieurii* F (ZLF) | > 250 | 45.8 ± 25.0 * | > 250 |
| *Z. zanthoxyloides* F (ZZF) | > 250 | > 250 | > 250 |
| *Chloroquine* | 0.43 ± 0.08 $^{\beta}$ | | |

F: fruits; L: leaves; SB: stem bark. HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol.

$IC_{50}$: concentration of extract needed to produce 50% of the *β*-hematin formation inhibition, Values are presented as mean ± SEM (n=3). *p<0.05, $^{\beta}$p<0.001 vs control using Mann-Whitney test.

**Table 3.** Cell growth inhibitory activities ($IC_{50}$) of the different extracts against A549, MCF7 and PC3 cancer cells

| Plants | $IC_{50}$ (µg/mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cell lines | A549 | | | MCF7 | | | PC3 | | |
| Solvents | HEX | DCM | MeOH | HEX | DCM | MeOH | HEX | DCM | MeOH |
| COL | > 250 | 206.1 ± 14.4* | 217.2 ± 39.5* | 51.4 ± 11.0 $^{\beta}$ | 84.4 ± 18.4 $^{\beta}$ | > 250 | 45.5 ± 62.4 $^{\beta}$ | 123.6± 1.8* | 50.0± 2.6 $^{\beta}$ |
| COBSB | > 250 | > 250 | > 250 | 31.6 ± 9.0* | > 250 | > 250 | 71.7 ± 1.5 $^{\beta}$ | 59.8 ± 3.0 $^{\beta}$ | > 250 |
| ECSB | 28.1± 6.3* | 111.0 ± 20.5* | > 250 | 40.3 ± 5.2 $^{\beta}$ | 21.6 ± 0.9* | > 250 | 204.7 ± 17.7* | 32.2 ± 6.1 $^{\beta}$ | > 250 |
| PMF | 32.5 ± 8.5 $^{\beta}$ | 53.0 ± 8.8 $^{\beta}$ | > 250 | 185.8 ± 5.5* | 61.1 ± 2.9 $^{\beta}$ | > 250 | 139.6± 21.2* | 66.2 ± 2.0 $^{\beta}$ | > 250 |
| PML | > 250 | > 250 | > 250 | > 250 | 146.2 ± 9.8* | > 250 | > 250 | 147.5 ± 3.7* | > 250 |
| PMSB | > 250 | 177.1± 612.5* | > 250 | 89.0 ± 2.9 $^{\gamma}$ | > 250 | > 250 | 63.0 ± 2.5 $^{\beta}$ | 217.3± 8.6* | > 250 |
| RHL | 127.8 ± 38.6* | 113.8 ± 3.5* | > 250 | > 250 | 40.3 ± 6.6 $^{\beta}$ | > 250 | > 250 | 64.4 ± 4.3 $^{\beta}$ | > 250 |
| ZLF | 55.3 ± 6.8 $^{\beta}$ | 67.3 ± 9.2 $^{\beta}$ | > 250 | 84.7 ± 5.8 $^{\gamma}$ | 174.0 ± 18.2* | > 250 | 49.5 ± 9.0 $^{\beta}$ | 58.1 ± 5.1 $^{\beta}$ | > 250 |
| ZZF | 105.0 ± 6.8 $^{\gamma}$ | 206.6 ± 11.5* | > 250 | 43.6± 6.5 $^{\beta}$ | 54.4 ± 8.5 $^{\gamma}$ | > 250 | 10.6 ± 1.0* | 93.2 ± 4.4 $^{\gamma}$ | > 250 |
| Doxo | 1.3 ± 0.3* | | | 0.7 ± 0.1* | | | 16.4 ± 2.9* | | |

F: fruits; L: leaves; SB: stem bark. HEX: *n*-hexane; DCM: dichloromethane; MeOH: methanol. Doxo = Doxorubicin. $IC_{50}$: concentration of extract causing 50% of cells death. Values are presented as mean ± SEM (n=3). *$p<0.05$, $^{\alpha}p<0.01$, $^{\beta}p<0.001$, $^{\gamma}p<0.0001$ vs control using Mann-Whitney test.


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## STATEMENT OF ETHICS

None needed

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS CONTRIBUTION

Stephanie T. Guetchueng investigation, writing-original draft, writing-reviewing and editing, data curation; Lutfun Nahar supervision, writing-original draft, data curation, writing-reviewing and editing; Kenneth James Ritchie supervision, writing-reviewing and editing, data curation; Fyaz M.D. Ismail supervision, writing-reviewing and editing, data curation; Andrew R. Evans writing-reviewing and editing, data curation; Alembert T. Tchinda writing-original draft; writing-reviewing and editing; Arrey P. Tarkang writing-original draft; writing-reviewing and editing, data curation; Emmanuel N. Nnanga writing-reviewing and editing; Satyajit D. Sarker supervision, writing-original draft, writing-reviewing and editing, data curation

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