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Comparative evaluation of antiplasmodial and cytotoxic Activities of *Anonidium mannii* (Oliv) Engl. et Diels leaves, trunk and roots

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Abstract
Anonidium mannii (Oliv) Engl. and Diels is an Annonaceae used in traditional medicine in many countries including Cameroon, in the treatment of cancer, malaria and rheumatism. Our study aims to evaluate and compare the antiplasmodial activity and study the cytotoxic of its methanolic extracts of the leaves, trunk (wood and bark) and roots (wood and bark).
The evaluation of antimalarial activity consisted of determining the percentage viability of 3D7 *P. falciparum* cells in the presence of our extracts by measuring the activity of lactate dehydrogenase (produced by the parasite) which is quantified by reading the absorbance at 620 nm indicating the number of parasites in the well and then determining the IC_{50} of our significantly active extracts. Cytotoxic activity was evaluated on the HeLa cells of the Tsd strain by quantifying the cells surviving exposure to our extracts by reading the fluorescence of quantified resorufin in a microplate reader.
Antimalarial activity showed that at a concentration of 50 µg/mL, the majority of the tested extracts gave significant antimalarial activity (percentage viability below 30%) against *P. falciparum* strain 3D7. Root wood showed the best activity (% viability = 1.7%; IC_{50} = 33.38 µg/mL). At up to 50 µg/ml, all the extracts showed a high rate of survival among the HeLa cells. No effect was observed with the leaf extract showing no activity (100% of survival).
These results justify the use of stem bark and leaves in the traditional pharmacopoeia for the treatment of malaria. However, since the roots wood presented the best activity, it would be more beneficial to suggest them to the local populations against this pathology after additional studies. The cytotoxic activity of the extracts on HeLa cells of the Tsda strain revealed that the methanolic extracts of leaves, stem bark, trunk wood, root bark and root wood possess low activity and could therefore be promising for the development of antimalarial molecules. This is first report on comparative antiplasmodial effect and cytotoxic study of the different parts of *A. mannii*.

Keywords: *Anonidium mannii*, antiplasmodial activity, cytotoxicity, *Plasmodium falciparum* 3D7, HeLa, medicinal plant

1. Introduction
Malaria is a parasitic disease that is transmitted by the female Anopheles mosquito. In 2012, there were approximately 247 million cases and over 619,000 deaths of malaria worldwide [1]. Children under 5 years of age account for 67% of malaria-related deaths worldwide, and more than 93% occur in sub-Saharan Africa [2], including 12.8% recorded in Cameroon in 2017 [3]. Geographical and economic inaccessibility to modern health care as well as the inadequacy of the health workforce are factors that result in more than 80% of the population in Africa relying on traditional medicine for the treatment of malaria [4].
In Africa, indigenous plants play an important role in the treatment of a variety of diseases as shown by Phillipson in 1995 [5]. A part of our research program consists in the analysis of the antiplasmodial activities of plants used by traditional healers to treat and cure malaria symptoms [6-8]; such a strategy allowed the selection of plants which are known to be used and obviates the limitations of blind screening.
In order to contribute to the enhancement of floristic biodiversity in general and in particular to the study of *Anonidium mannii* (Oliv) Engl. and Diels, a medicinal plant from Cameroon, we directed our research to the evaluation of the antiplasmodial activity of the different parts of

this plant, motivated on the one hand by its use in traditional medicine for the treatment of malaria [9, 10]. On the other hand, a literature review revealed few studies antiplasmodial and cytotoxic activity of *A. mannii* leaves, twigs and stem bark [9-11]. However, the phytochemical study, the antiprotozoal activity of the leaves and stem bark [10, 11], the antimycobacterial activities of the leaves and twigs [12]; the antioxidant, cytotoxic and antibacterial activities of the leaves [13, 14]; phytochemical screening and antioxidant activity in all parts (root wood, leaves, trunk bark, trunk wood and root bark) [15]; the toxicity (acute and subacute) and the evaluation of the antimicrobial activities of the leaves and stem bark (G, Ekounè *et al*., 2022) [16], as well as the antibacterial activity of the methanol extract of the leaves and roots of *A. mannii* [17] have already been studied. Consequently, the aim of this work was to evaluate and compare the antiplasmodial activity and study the cytotoxicity of the methanolic extract of *Anonidium mannii* leaves, trunk wood, stem bark, root wood, root bark in order to determine the parts displaying the best activity.

2. Materials and Methods

2.1 Plant material

The plant materials used consisted of leaves, trunk and roots of *Anonidium mannii*. They were harvested in October 2019 (raining season in Cameroon) in a forest of the village Nkolotou'outou, District of Sangmélima, Department of Dja et Lobo, South region of Cameroon. A botanist from the National Herbarium of Cameroon identified the plant by comparison with the botanical collection of D.W. Thomas N° 2188 registered in the same Herbarium under N° 50327/HNC.

2.2 Extraction

After harvesting, the barks were separated from the wood on the one hand and from the roots on the other. The parts obtained, namely the leaves, trunk wood, stem bark, root wood, root bark, were cut into small pieces, dried and then machine-pulverized into fine fibers. The preparation of methanolic extracts of the plant was carried out in accordance with the method described by Bidié *et al*. (2008) with some modifications [18]. Eight hundred grams (800 g) of shredded plant leaves were mixed with 5 L of 96% methanol; one thousand grams (1000 g) of shredded wood from the roots of the plant with 6 L of 96% methanol; one thousand one hundred grams (1100 g) of shredded wood from the trunk of the plant with 5 L of 96% methanol; one thousand one hundred grams (1100 g) of shredded bark from the roots of the plant with 6 L of 96% methanol; one thousand one hundred grams (1100 g) of ground bark from the trunk of the plant with 7 L of 96% methanol. The mixture obtained in each case was shaken using a spatula once a day for 2 days at room temperature (25 °C), to facilitate the dissolution of the compounds contained in the ground material; then the mixture was filtered three times through cotton wool and on 3 mm Wattman filter paper. Finally the filtrate was evaporated at 40 °C under reduced pressure using a rotary evaporator (Heidolph LABOROTA 4000); the crude extract obtained was weighed (15.2 g for leaves, 13.09 g for stem bark, 7.09 g for trunk wood, 40.57 g for root bark and 10.06 for root wood) and stored in a refrigerator (4 ºC) prior to further uses.

2.3 Malaria parasite

In this study, a cell line of malaria parasite was used, namely the *Plasmodium falciparum* strain 3D7, which was provided by the Biomedical Research Center of the Free University of Rhodes in South Africa.

2.4 Assessment of antiplasmodial activity

Activity against *Plasmodium falciparum* chloroquine-sensitive 3D7 strains was assessed following the procedure described by Desjardins *et al*. 1979 [19] with slight modifications as per Mbosso *et al*. (2018), Fouokeng *et al*. (2019), Mbosso *et al*. (2020) [7, 20, 21]. Briefly, a 4-8% parasitemia was used. Malaria parasites were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM hepes (Lonza). The medium was further supplemented with 5% Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2-4% hematocrit human RBC. The parasites were cultured at 37ºC under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 in sealed T25 or T75 culture flasks. Different extracts (*A. mannii* methanolic leaves extract (AMMle), *A. mannii* methanolic extract from the stem bark (AMMsb), *A. mannii* methanolic extract of the trunk wood (AMMtw), *A. mannii* methanolic extract of the root bark (AMMrb), *A. mannii* methanolic extract from the root wood (AMMrw)) solutions (60 µL) were added to the parasite cultures in 96-well plates and incubated in a 37 ºC CO_2 incubator. After 48 h, the plates were removed from the incubator. A volume of culture (20 µL) was removed from each well and mixed with 125 µL of a mixture of Malstat solution and NBT/PES solution in a fresh 96-well plate. The parasite lactate dehydrogenase (pLDH) activity was then measured in these solutions. A purple product forms when pLDH is present, and this product could be quantified by absorbance at 620 nm (Abs620). The Abs620 reading in each well was thus an indication of the pLDH activity and thereby of the number of parasites in the well. For each concentration of the extracts, the percentage of parasite viability and the activity of the pLDH in the wells of the well-treated compounds compared to the untreated controls were calculated. The test was performed in triplicate and the standard deviations were determined. Products that significantly reduced parasite viability at fixed concentration were used to determine the IC_{50}.

2.5 Cytotoxicity study

To assess the overt cytotoxicity, samples were incubated for 24 h, at a concentration of 50 µg/mL in 96-well plates containing HeLa cells (human cervix adenocarcinoma), maintained in a culture medium made of Dulbecco's Modified Eagle's Medium (DMEM) with 5 mM L-glutamine (Lonza), supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin/fungizone - PSF) cells for 24 h. HeLa cells were plated in 96-well plates at 2×104 cell per well. After an overnight incubation in a 5% CO_2 humidified incubator, 10-fold serial dilutions of compounds were added to the cultures (Triplicate wells; 200 µL final culture volume) and incubation continued for an additional 48 hours. The numbers of cells surviving drug exposure were quantified using the resazurin based reagent and resorufin fluorescence quantified ($Excitation_{560}/Emission_{590}$) in a multiwell plate reader [7, 20, 22].

2.6 Ethical considerations

Ethical clearance and research authorizations were obtained for the performance of our work at our various study sites. We certify that all the total extract and different fractions obtained in this work were used only for biological activity evaluation and cytotoxicity.

2.7 Data Analysis and Statistical Parameters

Microsoft Word 2013 was used for data entry, Microsoft Excel 2013 for making tables and diagrams. GraphPad Prism

program version 5.02 was used for data analysis.

3. Results and discussion

3.1 Antiplasmodial activity

The viability percentage of *Plasmodium falciparum* 3D7 cells and the standard deviation obtained for each sample is reported in Figure 1. Each of the 5 extracts was tested at a concentration of 50 µg/ mL, to determine the parasite viability and the activity of the (parasite lactate dehydrogenase) pLDH in the wells. The AMMle extract reduced the viability of *P. falciparum* 3D7 cells by approximately 21.8%, AMMsb by 18.3%, AMMtw by 4.4%, AMMrw by 1.7% while AMMrb presented a 75.7% viability.

Subsequently, IC_{50} of AMMle, AMMsb, AMMtw and AMMrw extracts were determined by graphical regression method on dose-response curves at a fixed-concentration of parasite (25 µg/mL). Promising antiplasmodial activities were obtained with IC_{50} of 36.16; 38.70; 41.97 and 33.38 µg/mL, for extracts AMMtw, AMMle, AMMsb and AMMrw respectively (Figure 2).

For comparison purposes, chloroquine (an antimalarial drug) was used as a standard with IC_{50} of 0,091µM.

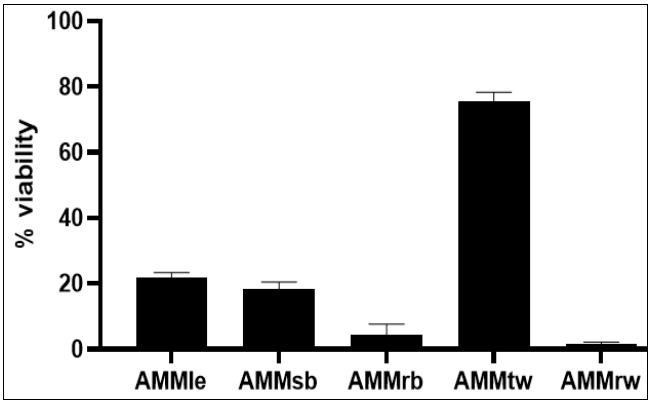

Fig 1: Percentage of *Plasmodium falciparum* 3D7 viability on *Anonidium mannii* methanolic extracts

% viability = Percentage viability; AMMle = *A. mannii* methanolic leaves extract, AMMsb = *A. mannii* methanolic extract from the stem bark, AMMtw = *A. mannii* methanolic extract of the trunk wood, AMMrb = *A. mannii* methanolic extract of the root bark, AMMrw = *A. mannii* methanolic extract from the root wood.

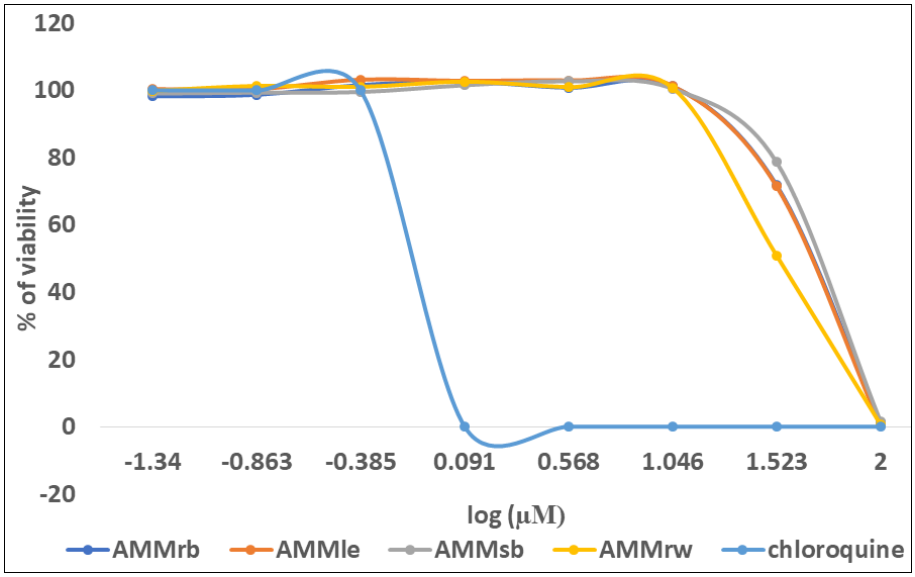

Fig 2: Dose-response curves of the anti-malarial assay of *Anonidium mannii* extracts. Positive control was pure chloroquine compound with an IC_{50} value of 0.091 µM

% viability = Percentage viability; AMMle = *A. mannii* methanolic leaves extract, AMMsb = *A. mannii* methanolic extract from the stem bark, AMMtw = *A. mannii* methanolic extract of the trunk wood, AMMrw = *A. mannii* methanolic extract from the root wood, Chloroquine = Reference antimalarial drug.

Total extract and fractions were tested for antiplasmodial activity against chloroquine-sensitive *Plasmodium falciparum* strains (3D7) using Chloroquine as drug reference. An extract or fraction is considered to be highly active when $IC_{50} \leq 5$ µg/mL [23]. Four of the five extracts were able to decrease the viability of *Pf*3D7 under 50% (38.46%) (Figure 1). These extracts showed a moderate activity with an IC_{50} of 36.16; 38.70; 41.97 and 33.38 µg/mL, for extracts AMMtw, AMMle, AMMsb and AMMrw respectively (Figure 2). These results suggest that hexane fraction contain compounds responsible for this activity, the presence of alkaloids. Indeed these different extracts are rich in alkaloids according to the work Mbosso *et al*. in 2022 [15]. The methanolic extract of the roots bark having only very slightly reduced the viability of these cells, with a percentage of 24% (less than 75%), has an insignificant activity. The phytochemical screening of this extract showed the presence of alkaloids as well as in the other parts tested [15]. Thus, this difference could be attributed to a lower alkaloid and terpene content in this part of the plant. We have oriented our choice on the evaluation of the antimalarial activity guided by the traditional pharmacopoeia BAKA in which the stem barks of *A. mannii* are used to treat malaria. Indeed our results showed that the methanolic extract of the stem bark of *A. mannii* reduces the viability of *P. falciparum* by 82%, with an IC_{50} of 41.97µg/ml. The results of our studies showed a similarity with the work of Fekam *et al*. in 2011 which showed that the methanol fractions of the leaves and twigs of *A. mannii* were very potent against *P. falciparum* of the W2 strain, with respective IC_{50} values of 2.84 µg/mL and 2.04µg/mL [9]; the difference in activity would be due to the different strains tested. This work is also close to that of Musuyu *et al*. in 2012 that showed that the aqueous extract of the stem bark of *A. mannii* has antiplasmodial activity on the K-1 strain of *P. falciparum*

resistant to chloroquine and pyrimethamine with an IC_{50} greater than 64 µg/mL [11]; and recently, the study of Kamdem *et al*. in 2022 on the dichloromethane-methanol (1:1) extract of *A. mannii* leaves also shown moderate activity with an IC_{50} of 50 µg/mL, against *Plasmodium falciparum* [10].

3.2 Cytotoxic activity

The 5 methanolic extracts of *A. mannii* were subjected to cytotoxicity tests. The microplates were read and the results for percent viability and standard deviation are shown in the bar graph (Figure 3).

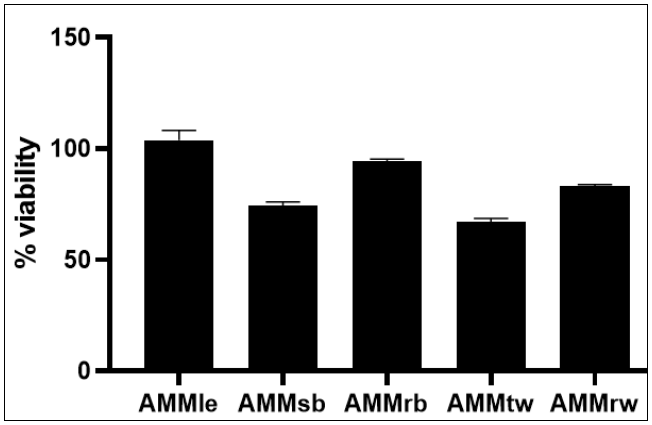

Fig 3: Percentage of HeLa cells viability on *Anonidium mannii* methanolic extracts

% viability = Percentage viability; AMMle = *A. mannii* methanolic leaves extract, AMMsb = *A. mannii* methanolic extract from the stem bark, AMMtw = *A. mannii* methanolic extract of the trunk wood, AMMrb = *A. mannii* methanolic extract of the root bark, AMMrw = *A. mannii* methanolic extract from the root wood.

This diagram illustrates the *in vitro* cytotoxic activity of the methanolic extracts AMMle, AMMsb, AMMtw, AMMrw and AMMrb on HeLa cells, tested at a concentration of 50 µg/mL. The AMMle extract didn't reduced cell viability by a relative 0%, followed by the AMMtw extract (5.8%), then the AMMrw extract (17.0%), then the AMMsb extract (25.8%) and finally, the AMMrb extract (33.1%). The extracts did not significantly reduce HeLa cell viability (> 50%) and therefore the IC_{50} was not determined for any of the extracts.

The diagram in Figure 3 shows the results of the *in vitro* cytotoxicity of methanolic extracts of *A. mannii*, tested at a concentration of 50 µg/mL on HeLa cells (from the human cervix). These results showed that the treatment of these cells with extracts of *A. mannii* would have affected cell viability. However, the cytotoxic effect is revealed by a mortality rate of 0 to 33.1%, which suggests that the toxicity of *A. mannii* extracts towards these human cells remains very low. The percentages of cell viability remained high (> 50%) for all tested extracts, their IC_{50} values were not assessed. Hence, extracts of leaves, stem bark, trunk wood, root bark, and root wood of *A. mannii* were considered non-cytotoxic. Dzoyem *et al*. in 2014 showed that the acetone extract of *A. mannii* leaves had a non-significant antiproliferative activity on Vero monkey kidney cells with an IC_{50} of 249 µg/mL [13], whereas the work carried out by Kuete *et al*. in 2013 showed that the methanolic extract of *A. mannii* leaves inhibited the growth of 8 different strains of multiresistant tumor cells by more than 50% at a concentration of 40 µg/mL with respective IC_{50s}: CCRF - CEM (17.33±2.27 µg/mL), CEM/ADR 5000 (16.44±76 µg/mL), MDA-MB-231 (12.65±1.49 µg/mL), MDA-MB-231-BCRP (32.02±3.16 µg /mL), HCT116P53+/+ (13.61±1.79 µg/mL), U87MG (22.25±2.76 µg/mL), U87MGΔE GFR (9.14±1.77 µg/mL), HepG2 (22.09 ± 2.42 µg/mL) [14].

4. Conclusion

The results of the present studies showed that antiplasmodial activity of the methanolic extracts of leaves, stem bark, trunk wood, and root wood could have moderate antiplasmodial activity. These results justify the use of stem bark and leaves in the traditional pharmacopoeia for the treatment of malaria. However, since the roots wood presented the best activity, it would be more beneficial to suggest them to the local populations against this pathology after additional studies. The cytotoxic activity of the extracts on HeLa cells of the Tsda strain revealed that the methanolic extracts of leaves, stem bark, trunk wood, root bark and root wood possess low activity and could therefore be promising for the development of antimalarial molecules. This is first report on comparative antiplasmodial effect and cytotoxic study of the different parts of *A. mannii*.

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6. Author contribution

Conceived and designed the experiments: JEMT. Performed the experiments: SNN SVF CNN. Analysed the data: GDM. Contributed reagents/materials/analysis tools: WD. Wrote the paper: JEMT. Validation and Supervision: JEMT GMMEL. Writing - Review & Editing, all authors.

7. Conflicts of interest

The authors have declared that there is no conflict of interest.

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