# Screening of Plant Extracts for Searching Antiplasmodial Activity

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**RÉSUMÉ**

L'objectif principal de cette étude consiste à isoler les molécules actives de plantes utilisées en médecine traditionnelle pour traiter le paludisme et ses symptômes.

197 plantes ont été collectées sous la base des enquêtes ethnobotaniques. Elles ont été séchées, broyées et macérées dans une solution alcoolique. L'extrait brut obtenu est passé au criblage biologique afin d'évaluer son activité antipaludique et sa toxicité (*in vitro* & *in vivo*).

Pour les plantes collectées à Madagascar : 15 extraits (soit 16%) préparés à partir de 91 plantes médicinales (dont 57 sont antipaludiques) testées présentent une activité antiplasmodiale intéressante avec une valeur de $CI_{50}$ inférieure à 10 µg/mL, leur cytotoxicité est faible avec une valeur de $CI_{50}$ située à plus de 50 µg/mL. Deux de ces extraits ont une $ED_{50}$ à moins de 100 mg/kg.

En ce qui concerne les plantes congolaises : cinq sur vingt six (soit 19.23%) plantes collectées seraient intéressantes.

**Mots-clés :**
Paludisme, Criblage, Plantes, Madagascar, Afrique

**ABSTRACT**

The aim objective of this study consists in isolating the active molecules from plants used in traditional medicine to treat malaria and its symptoms.

197 plants were collected following to the ethnobotanic investigations. Plants were dried, powdered and macerated in a hydroalcoholic solution. Resulting extracts have been assessed for *in vitro* & *in vivo* antimalarial and toxicity activities.

For the plants collected in Madagascar: 15 extracts (*i.e.* 16%) prepared from 91 medicinal plants (of which 57 are antimalarial) present an interesting antiplasmodial activity with a value of $IC_{50}$ less than 10 µg/mL, their cytotoxicity are weak with a value of $IC_{50}$ evaluated at more than 50 µg/mL. Two of these extracts have an $ED_{50}$ lower than 100 mg/kg.

With regard to the Congolese plants: five out of twenty six (*i.e.* 19%) collected plants would be interesting.

**Key words:**
Malaria, Screening, Plants, Madagascar, Africa

## INTRODUCTION

Malaria is an epidemic disease due to a parasite of the red blood cells called *Plasmodium* and which is transmitted by mosquito females called *Anopheles*. It is a pandemic disease at the origin of major problems of public health for the population living in more than 40% of the world territories **[1]**. Generally, in the countries of the sub-Saharan tropical zones, this disease kills out of the million people per year, and this mortality is very important in the children of less than five years. The World Health Organization estimates that 80% of population living in rural areas in developing countries depend on traditional medicine for their health care needs **[2]**. That is why the reason for which an international scientific collaboration between Madagascar and two countries of Africa was established with an aim of seeking antiplasmodial components isolating from plants used in traditional medicine to treat malaria. The results presented in this article are the fruits of this collaboration, thus the names of the studied plants are initially kept confidential to preserve the agreements in force.

## EXPERIMENTAL

### Plant materials

*Ethnobotanical studies*

An ethnobotanical survey has been realized to identify plants used in traditional medicine against malaria. Traditional practitioners and herborists (who sell dried plant material and advise people) have been interviewed with standardized questionnaires. These traditional practitioners treat malaria in function of symptomatology. During the survey, the following information was gathered for each medicinal plant collected: its vernacular name, the part used, preparation, administration and dosage.

*Plant collection*

Candidate plants were selected based on their local use as antimalarial or to treat its symptoms. They are collected at different locations in the surrounding forests of eight villages (East: Moramanga – Ambatondrazaka – Toamasina, West: Maevatanàna – Ampijoroa – Katsepy) of Madagascar. The most used plants have been collected and identified by personal at Botanic Park of Antananarivo. For each plant, a voucher herbarium specimen has been deposited in the Botanical Department of the Institute.

*Plant extraction*

Plants were air-dried at room temperature and powdered. For each species, 500 g of material was macerated three times for 72 h with 90% ethanol (EtOH) at room temperature. The combined extracts were filtered and solvents were evaporated to dryness under reduced pressure below 50 °C to yield a crude EtOH extract. Dry extracts were stored at –30°C until analysis.

### *In vitro* antimalarial assay

Quantitative assessment of in vitro antimalarial activity was determined by means of the microculture radioisotope technique based upon the method previously described by

Desjardins et al. **[3]** and modified by Ridley et al. **[4]**. The assay uses the uptake of [$^3$H]hypoxanthine by parasites as an indicator of viability.

*Parasite strain*
Continuous *in vitro* cultures of asexual erythrocytic stages of *Plasmodium falciparum* were maintained following the methods of Trager and Jensen **[5]**, on glucose-enriched RPMI 1640 medium, supplemented with 10% human serum at 37 °C. Plant extracts were tested on FCM29/C1, a chloroquine-resistant strain, as describe in our previous studies [Rafatro et al. **[6]**.

*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. **[7]**. 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 sulfate (Sigma, France) and 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 cell in medium (O+ group, 1% haematocrit) with 1% synchronous 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 in the percentage of inhibition, were calculated as 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 concentrations at which the parasite growth (=[$^3$H]hypoxanthine uptake) was inhibited by 50% ($IC_{50}$) was calculated by non-linear regression analysis processed on dose–response curves.

***In vivo* antimalarial tests**
*Suppressive parasitaemia assay*
The *in vivo* antimalarial activity of plant extracts was determined by the classical 4-day suppressive test against *Plasmodium yoelii sbsp nigeriensis* strain **[8]**. Briefly, adult male Swiss albino mice weighing 18 to 22 g were inoculated by intravenous (i.v.) route with $10^6$ *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 crude extract of *Cinchona* stem bark (500 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 × magnification).

*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 30% or more **[9]**. All extracts were firstly tested at daily doses of 500 mg/kg body weight. Then, the $ED_{50}$ representing 50% suppression of parasites when compared with untreated controls was estimated. Overall mortality was monitored daily in all groups during the period of 4-day test.

**Cytotoxicity assay**

*Cell culture*
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% $CO_2$.

*Test protocol*
Briefly, $5 \times 10^3$ 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 (Titertek Twinreader, Finland). Inhibition of cell proliferation was determined and expressed as percent of absorbance of NR extracted from control cells (defined as 100%).

***In vivo* toxicity study**

The acute oral toxicity was performed in mice. Plant extracts were administered orally via stomach tube after the animal was fasted for about 16 h prior to dosing. Extract preparations of the test plant were suspended in 30% DMSO (DiMethylSulfOxyde – Sigma, Germany; the same proportion was used when extracts were prepared for the *in vivo* antiplasmodial studies) in distilled water. A dosage of 2,000 mg/kg (five mice of each sex at each extract) was set for this study. The dose volume for 20-g individual animals was set at 0.5 ml/mice. The control group was treated with the vehicle (DMSO) alone. Mice mortality was observed for 14-day after the single administration.

## RESULTS

### Ethnobotanical information

The ethnobotanical investigations were carried out during the four forest expeditions: twenty one tradipraticians (two women and nineteen men) were interviewed. In traditional medicine, as in other areas of the world, the Malagasy tradipraticians evaluate the cure of malaria by the disappearance of the principal symptom of this disease among patients which is the fever. The remedies used by these healers were essentially made of plant decoctions, it was also noted during these investigations that the plants with bitter taste are frequently chosen for this purpose. These remedies are often prepared with branches (leaves and stems): the mode of preparation and the dosage are almost the same, in other words, a handful of plants in a gauge (1.5 L) of water which lets boil until the evaporation of the half quantity of the initial volume of water. Roots of the plants are rarely used. This preparation is divided into three parts to be given in the morning, the midday and the evening.

During this study, one hundred and ninety seven (197) plants were collected in Madagascar according to the following criteria:

1. Its use to treat malaria (to relieve the fever or plants with bitter taste), fifty seven (57) plants were collected;
2. As phytomedicines with virtues related or not to the treatment of malaria, there were forty five (45) plants;
3. Its systematic affiliation with the antimalarial plants but they are endemic species of Madagascar, they are ninety five (95) plants.

The Congolese plants were selected in this study because of their use in traditional medicine to treat malaria; there are twenty six (26).

***In vitro* antiplasmodial activity**

The dried plant powders were macerated in a hydroalcoolic solution (70°). Crude extracts were obtained after the filtration and the evaporation of this solution.

The percentage of inhibition of the parasitic growth following the study of a single concentration of 50 µg/ml of each extract was initially evaluated. An extract proves to be

interesting if this inhibition is total, in other words, this concentration causes an inhibition of more than 95% of the parasitic growth.

The screening of first intention with the plants of Madagascar gives the following results: fifteen plants (15) would be interesting (that is 7.61%), one hundred and thirty one (131) plants could be interesting with a percentage of inhibition between 30 to 95% and fifty one (51) plants would be not interesting because their percentage of inhibition is lower than 30%. Five (5) plants collected in Congo might be interesting (that is 19.23%), eleven (11) are not much interesting and ten (10) are not at all.

The inhibitory concentration ($IC_{50}$) of the crude extract of interesting plants was evaluated and the *in vitro* antiplasmodial results were classified as follow:

- For the $IC_{50}$ less than 10 μg/ml, the activity was considered good;
- If the $IC_{50}$ is between 10 to 50 μ g/ml, the extract was estimated active;
- If it is over than 50 μg/ml, the activity was undefined.

Of the ninety one (91) extracts of tested Madagascan plants, fifteen (15) present a good activity (that is 16.48%), seventeen (17) proved to be active and fifty-nine (59) are undefined.

***In vivo* antimalarial effect**

A preliminary study was carried out evaluating the percentage of inhibition of the parasitic growth following the administration of a single dosage of 500 mg/kg, the activity of the crude extract is interesting if it inhibits more than 60% of the parasitic growth.

The *in vivo* antiplasmodial results were classified as follow:

- For the $ED_{50}$ less than 10 mg/kg, the activity was considered very well;
- If the $ED_{50}$ is between 10 to 100 mg/kg, the extract was estimated good;
- From 100 to 1000 mg/kg, the activity was evaluated moderate;
- Over than 1000 mg/kg, the activity was undefined.

From the interesting Madagascan antimalarial plants resulted from the *in vitro* tests, twenty one (21) extracts were selected and tested. At a single dose of 500 mg/kg, three (3) extracts present a percentage of inhibition at more than 60% and seven (7) are at the value between 30 to 60%.

Then the efficacy dose to inhibit 50% of the *in vivo* parasitic growth was determined and two (2) plant extracts have a good activity.

**Toxicity outcome**

Along the *in vivo* test antimalarial, the mice mortality was recorded for the four days of treatment. During these studies, the number of death for each test never exceeded one of four mice tested for the daily dose of 500 mg/kg body weight, which is at the end of the treatment a total amount of 2 g of extract per mouse.

The toxicity of the plant extract was evaluated by two manners: on the one hand, the mice leukaemia cell line (named P388) and on the other hand, the acute toxicity carried out in mouse.

The observations gave the following results:

- Twenty (20) extracts were tested and seven (7, *i.e.* 35%) have a value of $IC_{50}$ less than 50 µg/mL;
- According to the acute toxicity tests in mice: of the eights (8) extracts from tested antimalarial plants, only one (1) extract kills the three quarter of animals. For the other extracts, the death rate does not exceed the 20%, this could be due to the amount of DMSO in the extract taken by the fasted animals.

## DISCUSSION

The major active compounds studied here were extracted from plants belonging to the families already published in the usual scientific revues. But, some essential points were evoked from this study:

1. These plants are bitter taste. This observation confirms the remarks of the tradipraticians and joined the scientific results quoted in the literatures: Euphorbiaceae **[10]**, Moraceae **[11]**, Annonaceae **[12]**, Loganiaceae **[13]**, Cucurbitaceae **[14]**;
2. They produce essential oils such as Canellaceae **[15]**, Pittosporaceae **[16]**;
3. The toxicity effect of the plant extracts is rather due to the molecules already described for this virtue once plant extract was taken at high dosage, such as alkaloids of the plants belonging to the families of Rubiaceae **[17]**, Rutaceae **[18]**, Apocynaceae **[19]**, Menispermaceae **[20]**; terpenes from plants of Simarubaceae **[21]**, Fabaceae **[22],** Asteraceae **[23]**.

Nevertheless, of new species belonging to the plant families not yet described in the literature were discovered and requiring a thorough study.

## CONCLUSION & PERSPECTIVE

During this investigation, it was observed that to discover a new and an original antiplasmodial molecule, it is very important to explore the non antimalarial but medicinal plants.

The relevant results evoked from this study are:

1. Two extracts of plants originate from Africa, resulting from traditional medicine but growing in Madagascar, are both active *in vitro* and *in vivo*;
2. The discovery of a Malagasy plant species, which the genus is endemic. It is an antimalarial plant which one of the interviewed tradipraticians was indicated. Its polar crude extract is active *in vitro* and that, according to our knowledge, any scientific study was still carried out in the field of malaria;

In prospect, five extracts of plants collected in the Republic Democratic of Congo and an extract of a Madagascar endemic plant are in the course of fractionation to isolate their active molecules.

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