Antiplasmodial Activities of Fractions and Natural Compounds from *Icacin a senegalensis* (Icacinaceae)

**Sénegal**

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Summary

Our project sought to contribute to the fight against malaria in Senegal. The main envisaged application remains both the treatment and prevention of malaria. We conducted our study on an antiplasmodial plant, *Icacina senegalensis*. The properties of this plant were discovered during a previous ethnopharmacological survey conducted in Senegal.

Our experiment in laboratory with *Icacina senegalensis* showed an inhibitory concentration, IC$_{50}$ of <5 µg / mL on the malaria parasite *Plasmodium falciparum* (pLDH method) and a selectivity index higher than 10 for mouse hepatocytes (Hepa 1-6) and human dermal fibroblasts (NHDF). This result shows interesting antiplasmodial activity without cytotoxicity. Moreover, our finished product could be an effective and well-tolerated treatment against malaria. Orientin, isoorientin, cis-clovamide and trans-clovamide were already identified from this plant during our study. It is interesting to emphasize the structural differences between these molecules and quinine and artemisinin. Furthermore, the compounds of interest can be produced by total or partial synthesis from the plant material and the antiplasmodial activity optimized by pharmacomodulation.

The raw material has been used by Senegalese traditional healers to cure malaria and no adverse effects have been reported from its use. The absence of toxicity observed by the traditional healers was later confirmed by haemolysis and *in vitro* cytotoxicity tests which showed good tolerance at a concentration 100-fold higher than those we used.

Our experience shows that the “ethno-bio-analytical” approach can provide access to more interesting results in the exploration of traditional medicine. This multidisciplinary approach combining ethnopharmacological surveys and bio-analytical assays deserves to be promoted for a better health in sub-Saharan Africa.

Background and Justification

Despite intensive efforts to control malaria, this disease continues to be one of the greatest public health problems in Africa. There were 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly one million deaths, mostly children under five years of age. One hundred and six (106) countries were still endemic for malaria in 2009 and of them 45 were within the WHO African region.

In Senegal, interesting progress against malaria has been achieved. The number of deaths due to malaria was estimated as 1678 in 2006 dropping to 577 in late 2009 according to data from the National Malaria Control Programme. However the burden of the disease
still remains a major public health threat. Almost all the most efficient antimalarial drugs are from natural sources (quinine and artemisinin derivatives). The global scope of malaria and the spread of drug-resistant *Plasmodium falciparum* makes the need for improved therapy urgent.

This experience was conducted in Senegal where malaria is still a public health problem and chemoresistance of *P. falciparum* is increasing. In this country, most of the antimalarials used officially are imported from Europa and Asia.

- Main issues involved:
  - Assessment and promotion of traditional medicine
  - Research of alternative treatment against malaria
  - Bio-guided fractionation of natural extracts
  - Multidisciplinary collaborative research
  - Partnership between scientists from academic fields and local traditional healers

### Description

We carried out chemical and bio-analytical methods to evaluate the biological properties of the plant in general and its antiplasmodial activity in particular.

Plants extracts were tested on strains of *P. falciparum* 3D7 Africa (chloroquine-sensitive) and 7G8 Brazil (chloroquine-resistant). Solid-liquid and liquid-liquid extractions techniques were carried out using organic solvents including methanol, methylene chloride and pentane. Samples tested were the defatted IM extract called IMd, the fraction obtained by liquid-liquid extraction using methylene chloride (IMD) and IMM which constituted the residual polar fraction.

We evaluated the haemolytic activity of extracts and fractions using a haemolytic agent, 5% sodium dodecyl sulphate (SDS) as a positive control. The negative control contained erythrocytes diluted (v/v) with the sterile malaria culture media (MCM). Haemoglobin content in the supernatants was determined by absorbance measurements at 538 nm in a microtitre plate spectrophotometer.

The cytotoxicities of both the extracts and the fractions on mouse liver cells (Hepa 1-6) and normal human dermal fibroblast (NHDF) were also assessed in vitro using the MTT colorimetric assay (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide). The assessment was performed in order to determine the selectivity index (SI). The SI was determined as the ratio between the IC$_{50}$ of cytotoxicity activity (concentration level
at which 50% of the parasite growth is inhibited) to that of anti-plasmodial activity. SI values were assessed with the NHDF cells (IC50 cytotoxicity) and on *P. falciparum* 3D7 (IC50 antiplasmodial activity). The IC50 was calculated using a nonlinear regression method. The results from the three independent experiments were reported as means with their standard deviations (SD).

The purification of the fractions was done using reverse semi-preparative chromatography using a C18 column type of 1 cm internal diameter. Biological techniques used comprised an assessment of the viability of the parasites in the presence of natural extracts at different concentrations. Thus, we used the enzymatic immunoassay technique based on the detection of *Plasmodium* Lactate Dehydrogenase (PLDH). PLDH is a metabolic enzyme produced during the parasite's development and is a good indicator of its viability.

Although time consuming, a bio-analytical and transdisciplinary approach was implemented in order to select and fractionate the natural active extracts on different biological models and *in vitro* cultures of *P. falciparum* respectively. The analytical approach adopted in this work enabled the isolation and the purification of active fractions from *Plasmodium* strains.

To optimize the biological activity and modulate the toxicity of such molecules, it appears useful to determine the chemical structure of the molecules previously purified. In our study, we highlighted the contribution of both modern chromatography and detection techniques such as Ultra High Performance Liquid Chromatography (UHPLC) with different detection systems.

Moreover, scientific studies such as ours will improve the methods of preparation of traditional medicines, providing scientific information on their potential toxicities and establish modern analytical methods for characterization. Finally, upon completion, this work will contribute to the enhancement of development of the Senegalese Pharmacopoeia.
Steps taken in the innovative and implementation process:

- Ethnopharmacological survey involving traditional healers and the local population
- Selection and identification of plants of interest
- Verification whether the selected plant is on the IUCN red list
- Production of bio-active extracts and fractions
- Haemolytic activity on red blood cells
- Antiplasmodial and cytotoxicity tests (*Plasmodium falciparum*, Hepa 1-6 cells, NHDF cells)
- Bio-guided chemical characterization of active extracts
- Separation, purification, isolation and identification of bioactive compounds.

**Results**

*Icacina senegalensis* (Icacinaceae) leaves (Fig. 1A) used against malaria and symptoms suggestive of possible malaria, were harvested in October 2007 in the plant's natural habitat in the province of Medina Sabakh (Senegal) (Figure 2).

![Figure 1: *Icacina senegalensis* at different development stages (A) and dried leaves (B).](image-url)
I. senegalensis revealed no haemolytic effect on red blood cells in vitro. Malaria Culture Media (MCM) was used as a negative control. These first results, which were not quantity-dependent, tended to confirm the traditional use, orally, by way of the decoctions and macerations of these natural extracts by the local and traditional tribes and without any side effects.
According to the WHO recommendations and previous works [32-34], anti-plasmodial activities of plant extracts were classified as follows: highly active extracts with IC$_{50}$ < 5 μg/mL, promising activity at 5-15 μg/mL, moderate activity at 15-50 μg/mL and inactivity at > 50 μg/mL.

With the aim of finding active subfractions and consequently identifying the substance(s) active against *P. falciparum*, many bio-analytical strategies were adopted, followed by a semi-preparative chromatographic method that was also used to produce five fractions from the active IM$^d$ fraction. The chromatographic profile is presented in Fig. 4.

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC$_{50}$ ± SD (μg/mL) on <em>P. falciparum</em></th>
<th>*IC$_{50}$ (Hepa 1-6) (μg/mL)</th>
<th>IC$_{50}$ (NHDF) (μg/mL)</th>
<th>*SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D7</td>
<td>4.7 ± 0.2</td>
<td>133 ± 20</td>
<td>&gt; 500</td>
<td>&gt; 106</td>
</tr>
<tr>
<td>7G8</td>
<td>8 ± 1</td>
<td>122 ± 4</td>
<td>&gt; 500</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>IMD</td>
<td>0.9 ± 0.2</td>
<td>447 ± 16</td>
<td>&gt; 500</td>
<td>&gt; 35</td>
</tr>
<tr>
<td>IMM</td>
<td>14.2 ± 0.7</td>
<td>32 ± 2</td>
<td>&gt; 500</td>
<td>&gt; 35</td>
</tr>
<tr>
<td>Chloroquine (nM)</td>
<td>44 ± 1</td>
<td>658 ± 14</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
</tr>
</tbody>
</table>

$^*$IC$_{50}$ = Inhibitory Concentration 50%; SI = selectivity index

**Table 1**: Antiplasmodial activity, cytotoxicity and selectivity indexes of samples IM$^d$, IMD and IMM (n=3).

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*Figure 4*: Chromatographic profile of defatted methanolic extract (IM$^d$).
Fractions F2A, F2B, F4A et F4B collected from IMD were tested on *P. falciparum* strains 3D7 and 7G8. The results obtained are presented in table 2:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>IC$_{50}$ 3D7</th>
<th>IC$_{50}$ 7G8</th>
</tr>
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<tbody>
<tr>
<td>F2A</td>
<td>0.73 ± 0.02 µg/mL</td>
<td>3.59 ± 0.24 µg/mL</td>
</tr>
<tr>
<td>F2B</td>
<td>4.52 ± 0.03 µg/mL</td>
<td>0.75 ± 0.03 µg/mL</td>
</tr>
<tr>
<td>F4A</td>
<td>3.78 ± 0.10 µg/mL</td>
<td>4.61 ± 0.41 µg/mL</td>
</tr>
<tr>
<td>F4B</td>
<td>3.53 ± 0.28 µg/mL</td>
<td>2.06 ± 0.41 µg/mL</td>
</tr>
</tbody>
</table>

Table 2: Antiplasmodial activity (IC$_{50}$) of fractions F2A, F2B, F4A and F4B (n=3) of defatted methanolic extract (IMD).

**Partnerships**

The project was conducted at University Cheikh Anta Diop in partnership with the Faculty of Pharmacy of University of Strasbourg in France. The weaknesses between partners were strengthened by an efficient communication approach using email exchanges, meetings and periodic reports on the project.

**Impact**

The project was conducted to a PhD degree level, obtained from Strasbourg University (France) However, the originality of the project and results obtained were emphasized during many national and international congresses, specialist meetings, etc.

The plant studied is a native species and could be cultivated on a large scale. The cultivation would provide the raw material and economic resources for local populations.

- This experience is an original approach from traditional use to scientific evaluation of the antiplasmodial activity of *Icacina senegalensis*.

- The route of administration is the traditional oral one and should allow a simple mode of administration and excellent bioavailability of the active ingredient(s).

- For the “user”, no adaptation will be required to use the product. However, it remains desirable that the cost of the finished product be as low as possible because of financial constraints and poverty in user countries. This status should allow them access to the new treatment. Furthermore, the solidarity fund grants or assistance to development will also promote free access to this product for disadvantaged populations.
• The extraction of active principles requires large amounts of expensive and toxic organic solvents. However the aqueous decoction could be an alternative “ecological” way of obtaining products of interest.

• A final advantage is the delay in the occurrence of chemoresistance of *Plasmodium* with this natural antimalarial.

• The part of the plant (endemic in Senegal) that contains active ingredient(s) is the leaf which allows intensive cultivation under industrial and sustainable development in the country that would be the main producer and supplier of the raw material. The opportunity to cultivate the plant on a large scale in Senegal would generate employment.

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**Replicability**

Until now there have been no policy implications. But the protection of traditional knowledge must be ensured by major political decisions.

Our experience has not brought about changes in legislation for the moment but it is urgent to define mechanisms and legislation to protect traditional medical knowledge which constitutes a national legacy. We hope that further discussion will be engaged in by the authorities on account of our findings.

No patents were applied for although a patent project existed but was never finalized.

• The innovative experience is important for other regions particularly in Africa where malaria remains a major public health threat.

• This plant grows in western and central Africa and therefore the raw material would be accessible in many countries.

• Our findings were exploited by Nigerian colleagues (University of Calabar, Nigeria) to run *in vivo* antiplasmodial activity on *Icacin a senegalensis* extracts in 2014.

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**Lessons Learned**

Obstacles faced and steps taken to overcome them:

• Availability of the plant material with the plant being harvested during and just after the rainy seasons;

• Financial resources to conduct all experiments necessary at the time required;
• Duration of the project to obtain the first results;

• Inconsistent governmental technical help during the research.

Preparing public perception for acceptance of the innovation:

• It is planned to share the results with the public after the finalization of the innovation (in vivo studies, analytical monograph for quality control, etc.);

• A communication strategy will be adopted in relation to this with the public health authorities.

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**Future Plans**

Plans for further improvement and expansion of the project:

• *In vivo* studies with natural extracts and hemi-synthetic molecules obtained from those isolated;

• Metabolomic approach to characterizing the natural extracts’ chromatographic profiles;

• Extract profiling according to the main seasons to ensure the quality of the plant in any given season (rainy season and dry season);

• Validation of an analytical monography for this plant to establish a modern quality control tool;

• A multidisciplinary team research on traditional medicine is being set up with a phytochemist, analytical chemist, biologist, toxicologist, biostatistician, etc.;

• Purification and identification of other active secondary metabolites of the plant;

• Research on other plants traditionally used to cure neglected diseases which constitute public health problems in Africa in general and in Senegal in particular.

Plans for collaborations and sharing of the results with other organizations/countries:

• Open access publication;

• Participation in national and international congresses;

• Organisation of national, regional and sub-regional workshops;

• Creation of open access database for sharing experiences and information exchanges between researchers and local population.
Publications


