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# A microfluorimetric method to screen marine products for antimalarial activity- Preliminary results

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## Abstract

Assessment of antimalarial activity with fluorescent dye when intercalating into the DNA was recently described. (1,2) The fluorescence avoids the handling of radio labelled compounds and the possibility to perform a high throughput assay. Like the incorporation of the $^{3}$H-hypoxanthine into the DNA (3), the fluorescence is linked to and reports the replication of the parasite.

The resurgence of the malaria in Africa, the spread of the resistance to currents antimalarial compel to identify new antimalarial head structures. Marine products were one of investigation fields for new antimalarials. We reported the use of the fluorochrome Picogreen® (1), fluorescent dye specific to nucleic acids currently used for ds-DNA quantification for identification of natural marine products with antiplasmodial activity.

**Key words**: Marine substance, antimalarial, *Plasmodium falciparum*, Picogreen®, fluorimetry.

## Introduction

The most effective antimalarial drugs available were derived from natural products isolated from terrestrial plants: quinine and artemissin.

Recent research proves that marine organisms also produce compounds with activity against malaria parasites (5,6). Manzamine A, β-carboline alkaloids, originally isolated from marine sponge *Haliclona* sp., significantly inhibits malaria parasite *in vitro* and *in vivo*. (7).

Most *in vitro* methods in these drug discovery screening used the gold standard technique: incorporation of $^{3}$H-hypoxanthine into the parasite DNA.(3). Very sensitive, this method allows testing many compounds in relatively short time and could be used in high throughput screening. However particular precautions and facilities are requested for handling radioactive compounds during the assay and their treatment after the assay.

Breakthrough in fluorimetry domain allows the DNA quantitative assessment by using fluorescent dye when intercalating them into DNA. Recent papers describe their implication in assessment of *Plasmodium* growth *in vitro*. Very sensitive, easy to use, they could be used in high throughput screening but they have the advantage to avoid using radiolabeled compounds.(1,2) Like the $^{3}$H-hypoxanthine method, the use of specific dyes for DNA reports

the parasite growth based on DNA replication.

Results presented use the intercalation of Picogreen® fluorochrome into the *Plasmodium* DNA to evaluate antiplasmodial activity of marine organisms extracts. PicoGreen® is an ultrasensitive fluorescent nucleic acid stain for measuring double-stranded DNA (dsDNA) in solution, and it enables the detection of quantities as low as 25 pg/mL of dsDNA with spectrofluorometer using fluorescein excitation and emission wavelengths (485nm/528nm). (1)

**Results and discussion:**

**Results**

***Sampling***

- 44 samples (listed in annex 1) were collected at Nosy-be during one field trip, into the International Cooperative Biodiversity Group programme. Sampling was carried out by the LME/CNRE group in collaboration with the Centre National de Recherches Océanographiques (CNRO) based at Nosy-be.

  11 were identified (complete scientific name) 25%
  21 identified to the genus level 48%
  12 were not identified 27%

- 3 sponges and 11 algae were collected at Toamasina and Toleara (annex 2) into the LME/CNRE programme. The 3 sponges collected and 5 out of the 11 algae collected are under identification.

**Antimalarial assay**

***Table 01:*** Antiplasmodial test results of samples collected at Nosy-be *Plasmodium falciparum* FCM29 strain was used for the test

| Samples | **% inhibition 50µg/mL** | Samples | **% inhibition 50µg/mL** |
|---|---|---|---|
| NB 11-04-00 | *27* | NB 11-04-23 | *0* |
| NB 11-04-01 | *Not Tested* | NB 11-04-24 | *11* |
| NB 11-04-02 | *20* | NB 11-04-25 | *0* |
| NB 11-04-03 | *N.T.* | NB 11-04-26 | *6* |
| NB 11-04- 04 | *N.T.* | NB 11-04-27 | *0* |
| NB 11-04-05 | *36* | NB 11-04-28 | *0* |
| NB 11-04-06 | *N.T.* | NB 11-04-29 | *0* |
| NB 11-04-07 | *N.T.* | NB 11-04-30 | *6* |
| NB 11-04-08 | *N.T.* | NB 11-04-31 | *2* |
| NB 11-04-09 | *N.T.* | NB 11-04-32 | *12* |
| NB 11-04-10 | *N.T.* | NB 11-04-33 | *1* |
| NB 11-04-11 | *9* | NB 11-04-34 | *6* |
| NB 11-04-12 | *9* | NB 11-04-35 | *1* |

| | | | |
|---|---|---|---|
| NB 11-04-13 | *N.T.* | NB 11-04-36 | *0* |
| NB 11-04-14 | *N.T.* | NB 11-04-37 | *0* |
| NB 11-04-15 | *0* | NB 11-04-38 | *0* |
| NB 11-04-16 | *4* | NB 11-04-39 | *11* |
| NB 11-04-17 /18 | *14* | NB 11-04-40 | *0* |
| NB 11-04-19 | *27* | NB 11-04-41 | *N.T.* |
| NB 11-04-20 | *21* | NB 11-04-42 | *N.T.* |
| NB 11-04-21 | *19* | NB 11-04-43 | *10* |
| NB 11-04-22 | *7* | NB 11-04-44 | *0* |

Quinine : IC50 = 3.5µg/mL

***Table 02:*** Results of **s**amples collected at Toleara, Fenoarivo, Toamasina *Plasmodium falciparum* FCM29 strain was used for the test

| **Marine algae** | Collect site | **% inhibition 50µg/mL** | | **Sponges** | Collect site | **% inhibition 50µg/mL** |
|---|---|---|---|---|---|---|
| TLR 2 | Toleara | 11 | | TLR 09 | Toleara | 5 |
| TLR 3 | Toleara | 20 | | TLR 10 | Toleara | 0 |
| Al 1 | Fenoarivo Atsinanana | 21 | | Tve | Toamasina | 0 |
| *Al 2* | Fenoarivo Atsinanana | 9 | | | | |
| *TLR 4* | Toleara | 65 | | | | |
| *TLR 6* | Toleara | 67 | | | | |
| *TLR 8* | Toleara | 57 | | | | |
| *TLR 7* | Toleara | 67 | | | | |
| Al 3 | Fenoarivo Atsinanana | 52 | | | | |
| *Al 4* | Fenoarivo Atsinanana | 60 | | | | |
| Al 5 | Fenoarivo Atsinanana | 65 | | | | |

Quinine : IC50 = 3.5µg/mL

**Discussion**

Climatic changes, impact of human activities on the environment, the green house effect and the warm of the planet are threats for the present global biodiversity. Marine fauna and flora as well as terrestrial are threatened.(8) Madagascar, fourth biggest island of the world has important coral reef zones and marine organisms but ill-known than its terrestrial living organisms. The threat on these marine resources due to pollution, human activity and climatic change urges its exploration for medicine use. Furthermore more than 70% of the samples presented here are not completely identified and under investigation.

CNARP/LME-CNRE, into the ICBG project, initiated recently (2005) the screening of marine organisms for antimalarial activity. Our results represent the antiplasmodial assay of all the samples collected during two missions. The fluorescent microfluorimetric method performed by a scientific team issued and realised in a developing country (1) is adequate for

a laboratory equipped with cell culture facility. It allows the screen of large quantity of samples in a relatively short time without the use of radio labelled compounds. We intend to test samples as many as possible before eventual species extinction.

The result is also an example of technology transfer and south-south cooperation between Panama and Madagascar, where malaria and other tropical diseases are matter of concern.

## Material and methods

### *Sampling*

Marine organisms (sponges and algae) were collected at Nosy-Be (coral reef of Sarodravay,Andrekareka, Nosy-Vorona, Dzamanjar), Toamasina (Foulpointe, Fenoarivo-Atsinanana) and Toleara.

### *Extraction*

Immediately after the collect, samples were cut, washed and partially dehydrated into a mixture of sea water/ethanol 50/50 for 24 – 48 hours. After filtration, the sampling were first macerated into methanol (3 x 24 hours) and followed by ethanol (80% - 70°C) extraction (3 x 1hour).

The active ethanol extracts was subjected to a Kupchan partitioning between water, hexane and dichloromethane to give 3 fractions of increasing polarity. 200µg of each fraction is prepared for antimalarial bioassay.

### Parasite culture

The chloroquine resistant strain (FCM29) of *P. falciparum* was provided by the Institut Pasteur de Madagascar. The strain was maintained *in vitro* by a modification of the Trager and Jensen method (4). The culture media consisted of standard RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated (56°C ; 1H) human type O+ serum, 25 mM NaHCO3, 2 mM glutamine, and 1 M HEPES (Sigma, St. Louis, MO). Culture was maintained in type AB+ human red blood cell suspensions collected from healthy local donors and prepared in citrate-phosphate-dextrose anticoagulant (Sigma, St. Louis, MO) at a hematocrit of 2%. The parasite density was maintained below 2% parasitemia under an atmosphere of a gas mixture containing $CO_2$, $O_2$, and $N_2$ - 5%, 5%, 90%, respectively - and at 37°C. For each experiment, sample of stock sorbitol-synchronised culture was further diluted in culture medium containing sufficient non infected type AB+ human erythrocytes to yield a final hematocrit of 2% and a parasitemia of 1%. All assays were carried out in 96-well microtiter plates.

### Fluorimetric susceptibility test

Synchronized ring form cultures (hematocrit 2% and parasitemia 1%) were used to test pure compounds or serial dilutions of extracts in 96-well culture plates. Culture of *P. falciparum* was placed in a humidified, air-sealed container, flushed with the gas mixture described earlier, and incubated at 37°C. Parasites were allowed to grow for a 48-hour incubation period, after which a 150µL aliquot of culture was transferred to a new 96-well flat bottom plate. Fifty microliters of the fluorochrome mixture, which consists of PicoGreen® (Molecular Probes, Inc., Eugene, OR), 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE buffer), and 2% Triton X-100 diluted with double-distilled, was then added to liberate and label the parasitic DNA. The plates were then incubated for 5−30 minutes in the dark. The fluorescence signal, measured as relative fluorescence units (RFU) was quantified with a

fluorescence microplate reader (FLx 800; Bio-Tek Instruments, Inc., Winooski, VT) at 485/20 nm excitation and 528/20 nm emission. Simultaneously, the RFU from positive and negative control samples were also performed.

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Annex 1
Collected Samples list – Nosy-Be

| ANDREKAREKA | |
|---|---|
| **1** | *Neomeris (vaventy)* |
| **2** | *Padina sp* |
| **3** | *Halymenia durvillaei (red alga)* |
| **4** | *Dictyota sp* |
| **5** | *Galaxaura sp* |
| **SARODRAVAY** | |
| **6** | *Halimeda macroloba* |
| **7** | *Xenia mucosa* |
| **8** | *Cinanchirella voeltzkowii* |
| **9** | *Sympodium (cf) coeruleum* |
| **10** | *Sympodium sp* |
| **11** | *Sarcophyton sp* |
| **12** | *Sinularia sp* |
| **13** | *Dysidea herbacea* |
| **14** | *Xestospongia sp* |
| **15** | *Xenia sp* |
| **16** | *Axinellidae (cf Axinella carteri)* |
| **17** | *Cynanchira sp ( ?)* |
| **18** | *Lobophytum depressum ou Sinularia abrupta* |
| **19** | *NI* |
| **20** | *NI* |
| **NOSY-VORONA** | |
| **21** | *Lobophytum sp* |
| **22** | *NI* |
| **23** | *Kallipilidion sp ( ?)* |
| **24** | *NI* |

| | |
|---|---|
| **25** | *Subergorgia sp ( ?)* |
| **26** | *Cladiella sp1* |
| **27** | *NI* |
| **DZAMANDJAR** | |
| **28** | *Halimeda opuntia* |
| **29** | *Avrainvillea obscursa* |
| **30** | *NI* |
| **31** | *Gracilaria sp* |
| **32** | *Dictyota sp* |
| **33** | *Gracilaria sp* |
| **34** | *Lynbia sp* |
| **35** | *NI* |
| **36** | *Sinularia sp* |
| **37** | *Lobophytum depressum* |
| **38** | *Sinularia sp* |
| **39** | *Udotea sp* |
| **40** | *Halimeda sp* |
| **41** | *NI* |
| **42** | *NI* |
| **43** | *Heteractis magnifica* |
| **44** | *NI* |

Annex 2
Collected Samples list – Toleara, Fenoarivo Atsinanana and Toamasina

| **Marine algae** | Collect site | **Sponges** | Collect site |
|---|---|---|---|
| TLR 3 (AB) | Toleara | EP2 BRV | Toleara |
| TLR 2 (AV) | Toleara | Ep/FT/Tve | Toamasina |
| *Acanthophora spicifera (AB)* | Fenoarivo Atsinanana | EP1 SRD | Toleara |
| Al 1 | Fenoarivo Atsinanana | | |
| *Phacellocarpus tristicus (AR)* | Toleara | | |
| *Chlorodesmys sp. (AV)* | Toleara | | |
| *Uva reticulata (AV)* | Toleara | | |
| *Chondrococcus sp. (AR)* | Toleara | | |
| *Hypnea sp* (AR) | Fenoarivo Atsinanana | | |
| FE 2 (AR) | Fenoarivo Atsinanana | | |
| FE 20 (AR) | Fenoarivo Atsinanana | | |

(AR): red alga (AV): green alga (AB): brown alga

## References


1. Corbett, Y., L. Herrera, J. Gonzalez, L. Cubilla, T. Capson, P. D. Coley, T. A. Kursar, L. I. Romero, and E. Ortega-Barria. 2004. A novel dna-based microfluorimetric method to evaluate antimalarial drug activity. *Am. J. Trop. Med. Hyg*. **70:**119–124

2. Martin Smilkstein, Nongluk Sriwilaijaroen, Jane Xu Kelly, Prapon Wilairat, and Michael Riscoe. 2004. Simple and Inexpensive Fluorescence-Based Technique for High-Throughput Antimalarial Drug Screening. *Antimicrob. Agents Chemother.* Vol. 48, No. 5, p. 1803–1806

3. Desjardins, R., G. Canfield, J. Haynes, and J. Chulay. 1979. Quantitative assessment of antimicrobial activity in vitro by a semiautomated microdilution technique. *Antimicrob. Agents Chemother*. **16:**710–718

4. Trager W, Jensen JB, 1976. Human malaria parasites in continuous culture. *Science 193:* 673–675.

5. Fidock D.A., Rosenthal P.J., Croft S.L., Brun R., Nwaka S. (2004) Antimalarial drug discovery: efficacy models for compound screening. *Nature reviews*, 3: 509-20.

6. Laurent D., Menou J.L., Frostin M., Parenty A., Videault A., Knibiehler M., Ausseil F., Dorin D., Doerig C., Schmitt S., Meijer L., Maurel S., Jullian V., Sauvain M. Antimalarial potentiality of xestoquinone, a proteine kinase inhibitor isolated from the Vanuatu marine sponge *Xestospongia* sp. 1er COST B22 Congress «Drug Development for parasitic Diseases», Anvers, Belgique, 22-24 novembre 2004.

7. Kenny K. H. Ang, Michael J. Holmes, Tatsuo Higa, Mark T. Hamann, and Ursula A. K. Kara (2000) In Vivo Antimalarial Activity of the Beta-Carboline Alkaloid Manzamine A. *Antimicrob Agents Chemother*., Vol 44(6): 1645–1649.

8. Chris D. Thomas, Alison Cameron, Rhys E. Green, Michel Bakkenes, Linda J. Beaumont, Yvonne C. Collingham, Barend F. N. Erasmus, Marinez Ferreira de Siqueira7, Alan Rainger, Lee Hannah, Lesley Hughes, Brian Huntley, Albert S. van Jaarsveld, Guy F. Midgley, Lera Miles, Miguel A. Ortega-Huerta, A. Townsend Peterson, Oliver L. Phillips & Stephen E. Williams (2004) Extinction risk from climate change. *Nature*, Vol 427, 145-148.