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# Anti-malarial Chemotherapy: potency guided screening from Brazilian plant species

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## Abstract

Malaria is a human parasitic disease caused by protozoa species of the *Plasmodium* genus. This disease has affected populations of the tropical and subtropical regions, specially in developing countries. Natural products have played a dominant role in the discovery of leads for the development of drugs to treat human diseases, and this fact anticipates that new antimalarial leads may certainly emerge from tropical plant sources. In this context, 16 Brazilian extracts and pure compounds from seven species were assayed against *Plasmodium falciparum* in order to find a new promising natural extracts or compounds. Despite of the high inhibition level of some extracts the results have shown the *n*-hexane extract from flowers of *P. lucaeanum* ($IC_{50}$ 3.50, SI 92.1) as the most promising fraction among all tested samples.

Keywords: Malaria, Plasmodium falciparum, natural products

## Introduction

Among all parasitic agents causing disease in humans, malaria still remains as one of the most serious and devastating tropical widespread diseases encountered by mankind, it continues to be a major threat to about 40 % of world's population, especially in the developing countries[1]. The WHO World Malaria Report 2008 estimated that there were approximately 247 million malaria cases among 3.3 billion people at risk in 109 countries where malaria is currently considered prevalent[2]. As malaria vaccines remain problematic, chemotherapy still is the most important weapon in the fight against the disease. However, almost all available drugs have been compromised by the highly adaptable parasite, and the increasing drug resistance of *Plasmodium falciparum* continues to be the main problem[3]. Natural product scaffolds have been the basis of the majority of current anti-malarial medicines. Compounds such as quinine and artemisinin were originally isolated from medicinal plants. In recent years, advances in screening technologies have allowed testing of millions of compounds from pharmaceutical diversity for anti-malarial activity[4]. Natural products may be lead compounds in themselves, or more likely may serve as hits that may be useful in providing pharmacophores/templates that can guide the design of potentially superior analogs and/or the mining of existing databases of synthetic and semi-synthetic compounds for previously untested and potentially active analogs[5]. Evaluation of plant extracts and natural products for antiplasmodial activity has resulted in the isolation and characterization of many dimeric chalcones, flavonoids, quinones and sesquiterpenes that have displayed anti-malarial properties. Indeed, the vast majority of the existing antimalarial chemotherapeutic agents are based on natural products, and this fact anticipates that new leads may certainly emerge from the tropical plant sources, since biological chemodiversity continues to be an important source of molecular templates in the search for antimalarial drugs[6]. It is often suggested that in herbal medicinal products, more than one active ingredient may be required for pharmacological activity. Although this is attractive to many in the herbal medicinal product field, the data supporting synergy of more than one sub-therapeutic ingredient is extremely sparse in the literature[7]. In many cases there is evidence of synergy, but the exact mechanisms have not been elucidated. The use of herbal teas preparations have been used as an alternative treatment for malaria in areas where people do not have access to, or cannot afford effective anti-malarials such as artemisinin combination therapy[8]. In addition, herbal medicines can sometimes be grown and produced locally, at lower cost, by or close to those who need them. This work aims to present the anti-malarial screening of non polar and polar fractions from seven different species extracts as well as pure isolated compounds from *Musa acuminata, Dorstenia arifolia, Eugenia rondifolia, Piper claussenianum, Piper truncatum, Piper lucaeanum* and *Pitcairnia corcovadensis* against *Plasmodium falciparum.*

## Material and Methods

### Plant material

Leaves, roots, stems and flowers from different plant species were collected in Rio de Janeiro, Brazil, Table 1. Dry powdered aerial plant parts were extracted in separatedly way consecutively with hexane, methanol and water for a period of 48h. After filtration, the solvent was removed under reduced pressure and the dried extracts were tested for antimalarial activity. Some active extracts were separated by chromatography into pure compounds.

see Table 1.

### Citotoxicity

The *in vitro* toxicity test was conducted using HepG2 A16 cell strain. For each experiment, the cells were seeded in 96-well plate, at a concentration of $4\times10^5$ cells/well. The plates were incubated for 24h

at 37°C in 5 % of $CO_2$ atmosphere. Cells were allowed to attach for 24h, the medium removed completely. After 24h, additional medium (100 µL) containing the tested compounds and extracts in different concentrations (1, 10, 100 e 1000µg/ml) were tested in triplicate. After incubation for 24 h with several concentrations of each compound and crude extract, the viability was evaluated by MTT (thiazolyl blue) assay. Briefly, MTT (Sigma) at 20.0 mg/ml was added to each well. After incubation for 1,5h at 37°C, the supernatant was removed and 100 µl of dimethylsulfoxide was added per well to dissolve the formazan crystals. The plates were read on a Thermomax Microplate Reader at 492 nm. IC50 values were determined as the drug and sample concentrations at 50% inhibition of the cell growth. The positive control was performed using chlroroquine as drug reference.

### *In vitro* Anti-plamodial Assay

Antiplasmodial activity was evaluated against the multi-drug resistant *Plasmodium falciparum* W2 strain (resistant to chloroquine), using the method HRP2 described by Noedl et al., 2002. The parasites were maintained in a continuous *in vitro* culture and the quantitative assessment of *in vitro* antiplasmodial activity was determined by means of the microculture radioisotope technique. The inhibitory concentration (IC50) represented the concentration that caused 50% inhibition in parasite growth, which was indicated by the uptake of the radio-labeled nucleic acid precursor, [3H]-hypoxanthine, by *P. falciparum* K1 strain maintained on human red blood cells *in vitro*. The definition of the antiplasmodial activity used was: $IC_{50}$ < 1 µg/ ml - strong activity; 1-15 µg/ml active, 15.1–25 µg/ml – moderate activity; 25.1-50 µg/ml - mild activity and $IC_{50}$ > 50 µg/ml - inactive. Chloroquine was used as standard.

### Selectivity Index (SI)

For the samples considered actives, the selectivity index was calculated. The selectivity index (SI) is defined as the ratio of the HepG2 toxicity to the anti-plasmodial activity and is determined by dividing the $LC_{50}$ values for the HepG2 by the $IC_{50}$ value for *P. falciparum*. The extract with higher selectivity (high SI value) indicates potentially safer therapy.

### Samples Solubility

All the samples were diluted in DMSO in a concentration of 50mg/ml.

## Results and Discussion

*In this study seven different Brazilian plants were investigated about their antiplasmodial activity against P. falciparum* W2. Out of 16 plant extracts and pure compounds evaluated, 6 were active against *P. falciparum* strain W2 ($IC_{50}$ 1-15 µg/ml), 2 were moderately active ($IC_{50}$15.1–25 µg/ml) while the remaining 8 were considered inactive ($IC_{50}$ > 50 µg/ml). Four fractions and one pure compound were of particular interest since they had $IC_{50}$ values < 10. The leaf-fraction (1-7) ($IC_{50}$ 5.22), chromatographed from *n*-hexane *E. rotundifolia* extract, the *n*-hexane extract of *P. lucaeanum* stems ($IC_{50}$ 7.99), the *n*-hexane extract of *P. lucaeanum* flowers ($IC_{50}$ 3.50), *n*-hexane extract of *P. claussenianum* flowers ($IC_{50}$ 9.57) and 2', 6'-dihydroxy-4'-methoxychalcone ($IC_{50}$ 9.66) isolated from methanol extract of *P. claussenianum*. The most promising extracts in this assay were the non polar extracts. Despite of the lowest cytotoxicity $CC_{50}$ values > 500 µg/mL, the polar fractions of 4 selected species did not display a good activity in front of *P. falciparum*.

see Table 2.

Actually, although the methanol extracts of *D. arifolia* were considered the safest fractions $CC_{50}$ values > 1000, no activity was detected in these samples as is shown in the Table 02. The non polar fractions from hexane extract showed $CC_{50}$ values between 23.81 to 472.60 µg/mL. The parasite growth inhibition was assayed in concentrations of

25 µg/mL and 50 µg/mL of crude extracts and pure compounds, Table 3. The *n*-hexane flower extract obtained from *P. claussenianum* was able to inhibit 92 and 95% of the parasite growth, respectively. The chalcone 2', 6'-dihydroxy-4'-methoxychalcone, the chemical marker of methanol flower extract was able to inhibit 95 and 98%. Curiously, the methanol extract did not show a good activity even though the chalcone is the major compound in the extract yielding about 2.0% in the constituents mixture.

see Table 3.

With exception of the *n*-hexane flower extract, the non polar extracts obtained from leaves and branches of *P. lucaeanum* were able to inhibit 100.0% of the parasite growth in both concentration. The non-polar fractions obtained from *E. rotundifolia* had also high inhibition percentage when compared to the polar MeOH extract, showing about 2.0 fold activity. The growth percentage reduction was 69.0/ 87.0% and 30.0/ 41.0%, respectively for non-polar and polar fractions. The activities of fractions from *M. acuminata* and *D. arifolia* were very low or zero. Both species extracts were obtained using polar solvents in the extraction procedure. One of the lignans isolated from *P. truncatum* was active against the tested parasite. The eudesmine had a growth percentage reduction of 69.0 and 77.0% in 25 g/mL and 50 g/mL. However, another lignan was just slightly active achieving 10.0 and 15.0 % of the parasite inhibition in 25 µg/mL and 50 µg/mL, respectively. The selectivity index is correlated to the required safety to serve as an anti-malarial drug. Moderate and high selectivity levels were found to *n*-hexane fraction of leaves from *E. rotundifolia* (SI 29.9); *P. lucaeanum* (SI 30.6) and (SI 90.1) for hexanic fractions of leaves and flowers, respectively. The results suggest these non-polar fractions as the safest samples for growth inhibition of the parasite, once the action is toward the parasite inhibition instead of the cell damage. Despite of the considerable activity of *E. rotundifolia* leaf-fraction (1-7) ($IC_{50}$ 5.22) and *n*-hexane extract from flowers of *P. claussenianum* ($IC_{50}$ 9.57) these samples were considered cytotoxic, thus not acting selectively as antimalarial.

see Table 4.

The results suggest the *n*-hexane extracts of *P. lucaeanum* as the most promising extract among the studied species. The good inhibition level and selectivity displayed by encourage the continuous investigation if the involved compounds and/or synergism are responsible for the antimalarial activity. As it is known, in non-polar extracts many terpenes could be acting against on the parasite growth. Literature survey reports the influence of mono, sesqui and complex terpenes on the inhibition of *Plasmodium* growing, suggesting the importance of these compounds. This study can lead us to the bioassay-guided isolation of bioactive principles that could be undertaken to provide new anti-parasitic drugs or lead compounds for anti-malarial drug development.

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| Botanical name | Parts collected |
|---|---|
| *Dorstenia arifolia* | Leaves, roots |
| *Eugenia rotundifolia* | Leaves |
| *Musa acuminata* | Flowers |
| *Piper claussenianum* | Flowers |
| *Piper lucaeanum* | Leaves, stems, flowers |
| *Piper truncatum* | Leaves |
| *Pitcairnia corcovadensis* | Leaves |

Table 1: list of plant species colleted

| **Samples** | **$CC_{50}$ (µg/ml)** | **$CI_{50}$ (µg/ml)** *P. falciparum* W2 | **SI** |
|---|---|---|---|
| *P. claussenianum* | | | |
| Flowers/Hex. | 23.8 | 9.57 | 2.48 |
| Flowers/MeOH | 592.8 | 9.66 | 61.3 |
| | | | |
| *E. rotundifolia* | | | |
| Leaves/Hex. | 472.6 | 15.78 | 29.9 |
| Leaf-fraction*/Hex. | 42.7 | 5.22 | 8.1 |
| | | | |
| *P. lucaeanum* | | | |
| Leaves/Hex. | 438.5 | 14.30 | 30.6 |
| Branches/Hex. | 128.8 | 7.99 | 16.1 |
| Flowers/Hex. | 322.4 | 3.50 | 92.1 |
| | | | |
| Chloroquine | 311 | 0.780 | 398 |

Table 2. Cytotoxicity, antiplasmodial activity plus selectivity indices of extracts of selected Brazilian rain forest plants

*Leaf-fraction was obtained from chromatographed fractions of Leaves hexane extract from *E. rotundifolia*.

| Samples | % Red. | |
|---|---|---|
| | 25µg/ml | 50µg/ml |
| ***P. claussenianum*** | | |
| Flowers/Hex. | 92 | 95 |
| Flowers/MeOH | 0 | 52 |
| chalcone | 95 | 98 |
| ***M. acuminata*** | | |
| Flowers/MeOH | 0 | 23 |
| Flowers/Aqueous residue | 32 | 42 |
| ***D. arifolia*** | | |
| Leaves/MeOH | 0 | 0 |
| Roots/MeOH | 0 | 0 |
| ***E. rotundifolia*** | | |
| Leaves/Hex. | 69 | 87 |
| Leaves/MeOH | 30 | 41 |
| Leaf-fraction* | 66 | 76 |
| ***P. lucaeanum*** | | |
| Leaves/Hex. | 100 | 100 |
| Branches/Hex. | 100 | 100 |
| Flowers/Hex. | 74 | 100 |
| ***P. corcovadensis*** | | |
| Leaves/Hex. | 42 | 81 |
| ***P. truncatum*** | | |
| Lignan 1 | 10 | 15 |
| Eudesmine | 69 | 77 |
| Chloroquine | 100 | 100 |

Table 3. Antimalarial activity of pure compounds and crude extracts of selected brazilian plants.

*Leaf-fraction was obtained from chromatographed fractions of Leaves hexane extract from *E. rotundifolia*.

| Samples | Classification |
|---|---|
| ***P. claussenianum*** | |
| Flowers/Hex. | Active |
| Flowers/MeOH | Inactive |
| chalcone | Active |
| ***M. acuminata*** | |
| Flowers/MeOH | Inactive |
| Flowers/Aqueous residue | Inactive |
| ***D. arifolia*** | |
| Leaves/MeOH | Inactive |
| Roots/MeOH | Inactive |
| ***E. rotundifolia*** | |
| Leaves/Hex. | Moderately active |
| Leaves/MeOH | Inactive |
| Leaf-fraction* | Active |
| ***P. lucaeanum*** | |
| Leaves/Hex. | Active |
| Branches/Hex. | Active |
| Flowers/Hex. | Active |
| ***P. corcovadensis*** | |
| Leaves/Hex. | Inactive |
| ***P. truncatum*** | |
| Lignan 1 | Inactive |
| Eudesmine | Active |
| Chloroquine | Very active |

Table 4. Antimalarial activity classification of the investigated samples against the strain W2 (chloroquine resistent) of *P. falciparum*

*Leaf-fraction was obtained from chromatographed fractions of Leaves hexane extract from *E. rotundifolia*.