In vitro Antimalarial Activity of 11 Terpenes Isolated from Ocimum gratissimum and Cassia alata Leaves. Screening of Their Binding Affinity with Haemin

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Abstract

Eleven terpenes extracted from Cassia alata and Ocimum gratissimum leaves were screened for in vitro antimalarial activity against Plasmodium falciparum and for their binding affinity with haemin in ethylene Glycol-water 3:7 solvent. Nine terpenes have promising antimalarial activity with IC₅₀ values below 1µg/ml; two terpenes showed a good activity with IC₅₀ values below 4µg/ml. All the terpenes bind strongly with haemin as shown by variation of absorbance of the haemin at λ 600nm in UV-Visible spectrum.

Keywords: Cassia alata, Ocimum gratissimum, antimalarial activity, terpenes, binding affinity, haemin

1. Introduction

Many studies have been reported on the in vitro antimalarial activity of terpenes (Kalauni et al., 2006; Suksaran et al., 2006; Chukwejeku et al., 2005), on biological activities of Ocimum gratissimum essential oil (Ueda-Nakamura et al., 2006; Tchoumbougnang et al., 2005; Usip et al., 2006) and on antimicrobial activity of aqueous and ethanol extracts of Cassia alata (Somchit et al., 2003; Villasenor et al., 2002; Ranganathan et al., 2000).

In addition, the main antimalarial mode of action of quinolines (Chou et al., 1980; David & Sullivan, 2002; Sugioaka et al., 1987) and artemisinin derivatives whose structures are totally different with those of quinoline alkaloids (Kamnan et al., 2002; Krishna et al., 2004; Meshnick, 2002) has been established to be their inhibition of haemin polymerisation through their binding with haemin.

In the present work, we aim to evaluate in vitro the antimalarial activities of eleven terpenes isolated from Cassia alata (four) and Ocimum gratissimum (seven) given that terpenes are easily extractible and these leaves contain high levels of terpenes.

We carried out also qualitative studies of their binding affinity with haemin in order to get first knowledge of their mode of action; because the detection of haemin-binding properties of molecules could be used as a preliminary test for antimalarial activity (Steel et al., 2002).

2. Materials and Methods

2.1 Plant Materials

Plants were collected in Kinshasa/ Kisenso, DR Congo and were authenticated by the Herbarium service of Department of Biology, University of Kinshasa where voucher specimens are preserved. The leaves were air dried at room temperature for 20 days and then grinded with pestle and mortar.

2.2 Extraction of Terpenes

Extraction of terpenes was carried out according to the general procedure described by Bruneton (Bruneton, 1993). The dried and grinded leaves (200g) were macerated during 7 days in CH₂Cl₂ (2x2.5l) at room temperature. CH₂Cl₂ extract was suspended in EtOH-H₂O 3:7 mixture and then extracted with Petroleum ether (60-80°C) to afford fraction A after concentration under reduced pressure. EtOH -H₂O fraction was subjected to evaporation under reduced pressure to give a residue which was then dissolved in MeOH. The fraction B was obtained after evaporating MeOH. Fractions A and B were combined to give the terpenes extracts which were
separated by preparative TLC on precoated silica gel 60F254 plates (Merck) using EtOAc-Petroleum ether 4:1. Four terpenes (TCA1-TCA4) were isolated from Cassia alata and seven (TOG1-TOG7) from Ocimum gratissimum. Individual terpen was rechromatographed on silica gel column using the same solvent system. All isolated compound had a positive response to the Liebermann test (concentrated H₂SO₄ in mixture with acetic anhydride).

2.3 Antimalarial Activity

The in vitro assays were conducted by using the micro dilution technique of Desjardin (Desjardin et al., 1979). The P. falciparum parasites were derived by direct visualization and micro manipulation from fresh patient isolates. The test compounds were initially dissolved in EtOH:H₂O mixture (1:3) or in DMSO and diluted 100-fold in RPMI 1640 culture medium, supplemented with 25mM Hepes and 32mM NaHCO₃. These solutions were diluted in 10 different concentrations. The parasites were exposed to different dilutions of each compound for 48h and incubated at 37°C. Direct estimation of parasite growth inhibition was used and it was based on direct reading of smears made in 24-well, flat-bottomed plates to estimate growth and evolution stages of the parasites (Bemoit et al., 1996).

Parasitaemia and parasite stage were determined after 48h of contact between extracts and parasites. Concentration-response data were analyzed by nonlinear regression logistic dose-response model and IC₅₀ values for each compound were calculated.

2.4 Binding Affinity with Haemin

Propylene glycol-H₂O 3:7 was used as solvent for the study of binding affinity of all terpenes with haemin, owing to the fact that haemin forms dimers in aqueous media at pH< 9. In all experiments pH and haemin concentrations were maintained constant (pH 10.75; 0.3 × 10⁻⁵ = 2µg/ml). Under these conditions, haemin exists only in monomeric form. UV – Visible spectra of haemin were recorded between 420 and 700 nm using ZUZI® UV – 4200 spectrophotometer in presence of increasing concentrations of terpenes (0.02 to 2 mg/ml). A decrease of haemin absorbance at its λₘₐₓ (600 nm) indicates binding affinity of terpen with haemin. The binding affinity was estimated as the absorbance difference between haemin solution and haemin solution in presence of the highest concentration of terpen (2 mg/ml).

3. Results and Discussion

The results of the antimalarial activities of isolated terpenes (Table 1) show that the 4 terpenes from Cassia alata (TCA1 – TCQ4) and 5 from Ocimum gratissimum (TOG1, TOG2, TOG5, TOG6 and TOG7) are the very active against Plasmodium with all their IC₅₀ below 1µg/ml. TOG3 and TOG4 are active with IC₅₀ values <4µg/ml. The unceasingly crescent interest of terpenes is due to their high antimalarial activities. The IC₅₀ values recorded for many of them are around 0.1 to 3.5µg/ml (Kalauni et al., 2006; Suksarman et al., 2006; Chukwejeku et al., 2005; Jullian et al., 2005; Ma et al., 2005).

Table 1. Antimalarial activity of isolated terpenes

| Plant              | Terpene | Rf   | IC₅₀ (µg/ml) |
|--------------------|---------|------|--------------|
| Cassia alata       | TCA1    | 0.35 | 0.94         |
| Cassia alata       | TCA2    | 0.48 | 0.23         |
| Cassia alata       | TCA3    | 0.55 | 0.44         |
| Cassia alata       | TCA4    | 0.65 | 0.52         |
| Ocimum gratissimum | TOG1    | 0.06 | 0.32         |
| Ocimum gratissimum | TOG2    | 0.14 | 0.27         |
| Ocimum gratissimum | TOG3    | 0.21 | 1.41         |
| Ocimum gratissimum | TOG4    | 0.37 | 3.96         |
| Ocimum gratissimum | TOG5    | 0.47 | 0.44         |
| Ocimum gratissimum | TOG6    | 0.59 | 0.65         |
| Ocimum gratissimum | TOG7    | 0.87 | 0.52         |
| Quinine            |         |      | 0.1          |

In addition, terpenes are major components of essential oils which have various therapeutic virtues, justifying their use in traditional medicine. The main constituents of Ocimum gratissimum essential oil Eugenol,
phellandrene, thymol, limonene (Ueda-Nakamura et al., 2006; Tchoumbougang et al., 2005) are known to possess many biological activities (Lahlou et al., 2004; Interaminense et al., 2005; Usip et al., 2006). *Cassia alata* is used in traditional medicine in various regions of the world; its inhibition activity on the growth of larvae of *Chrysoma megacephala* has been reported (Kumarasinghe et al., 2002). To our knowledge, this study is the first to report an antimalarial activity of the terpenic fraction of *Cassia alata*.

The results mentioned in Table 2 show that all terpenes studied have a binding affinity with the haemin characterized by a diminution of haemin absorbance at the $\lambda_{\text{max}}$ (600 nm) when terpenes concentration increases (Figures 1 and 2).

### Table 2. Binding affinity of isolated terpenes with haemin

| Terpene | Rf | Biding affinity $\Delta A$ |
|---------|----|--------------------------|
| TCA1    | 0.35 | 0.089                   |
| TCQ2    | 0.48 | 0.115                   |
| TCA3    | 0.55 | 0.104                   |
| TCA4    | 0.65 | 0.091                   |
| TOG1    | 0.06 | 0.089                   |
| TOG2    | 0.14 | 0.092                   |
| TOG3    | 0.21 | 0.076                   |
| TOG4    | 0.37 | 0.079                   |
| TOG5    | 0.47 | 0.087                   |
| TOG6    | 0.59 | 0.080                   |
| TOG7    | 0.87 | 0.080                   |

$\Delta A = AH - AH + T$ with $AH$ = haemin solution absorbance; $AH + T$ = haemin + highest concentration of terpene solution absorbance.

This interaction with haemin could explain the high antipaludic activity recorded for this class of compounds. Indeed, Chauhan et al. (2002) have shown that a terpene like artemisinin inhibits the haemin polymerization in
hemozoin through its ability to form complexes with haemin. Paitayatat et al. (1997) have correlated antimalarial activity of artemisinin derivatives with their binding affinity with ferrirrprotoporphyrin IX (haemin). The fall in haemin absorbance at 600 nm is due to the interaction between porphyrin core of haemin and antimalarial compound. Because, an interaction by charge transfer which could take place between the central iron atom and the antimalarial could have been observed on UV part of haemin spectrum. So, the detection of haemin-binding properties of molecules could be used as a preliminary test for antimalarial activity (Steel et al., 2002).

4. Conclusion
Terpenes from Cassia alata and Ocimum gratissimum are very active compounds in malaria treatment. The qualitative studies of their binding affinity with haemin point to fact that they act on Plasmodiums through a binding with haemin.

Further investigations required determination of the structures of isolated compounds and quantitative studies of their binding affinity to haemin in order to determine they complexes formation constants.

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