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

Kayembe J. S.[1], Taba K. M.[1], Ntumba K.[1] & Kazadi T. K.[1]

[1] Department of Chemistry, Faculty of Sciences, University of Kinshasa, Democratic Republic of Congo

Correspondence: Kayembe J. S., Department of Chemistry, University of Kinshasa, Democratic Republic of Congo. E-mail: jeanksm@unikin.cd

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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 IC_{50} values below 1µg/ml; two terpenes showed a good activity with IC_{50} 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; Suksarman 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; Sugioka 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 CH_2Cl_2 (2x2.5l) at room temperature. CH_2Cl_2 extract was suspended in EtOH-H_2O 3:7 mixture and then extracted with Petroleum ether (60-80°C) to afford fraction A after concentration under reduced pressure. EtOH -H_2O 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 rechromatographied on silica gel column using the same solvent system. All isolated compound had a positive response to the Liebermann test (concentrated H_2SO_4 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:H_2O mixture (1:3) or in DMSO and diluted 100-fold in RPMI 1640 culture medium, supplemented with 25mM Hepes and 32mM $NaHCO_3$. 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 IC_{50} values for each compound were calculated.

2.4 Binding Affinity with Haemin

Propylene glycol-H_2O 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 IC_{50} below 1µg/ml. TOG3 and TOG4 are active with IC_{50} values <4µg/ml. The unceasingly crescent interest of terpenes is due to their high antimalarial activities. The IC_{50} 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	IC_{50} (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 λmax (600 nm) when terpenes concentration increases (Figures 1 and 2).

Table 2. Biding affinity of isolated terpenes with haemin

Terpene	Rf	Biding affinity ΔA
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

ΔA = AH – AH+T with AH = haemin solution absorbance; AH+T = haemin + highest concentration of terpene solution absorbance.

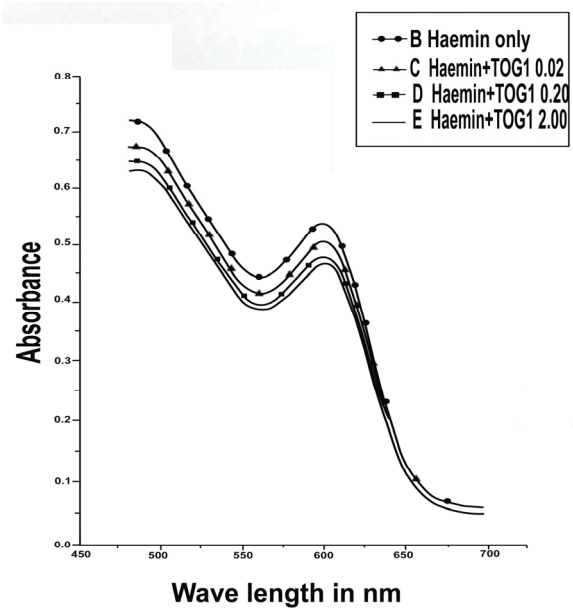

Figure 1. Haemin spectrum in presence of increasing concentrations of TOG1

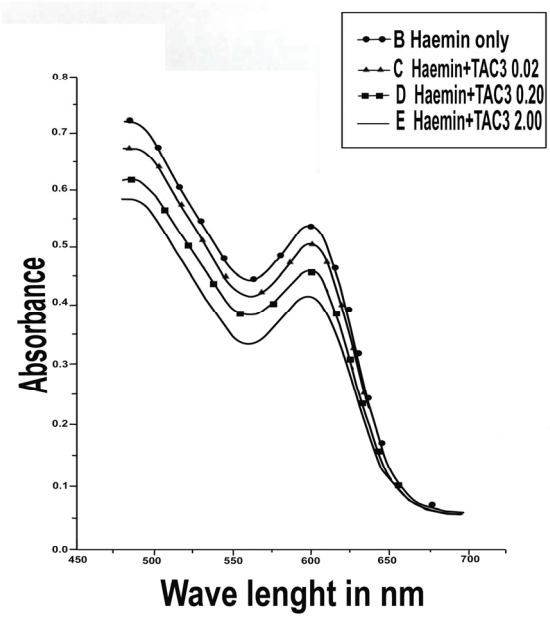

Figure 2. Haemin spectrum in presence of increasing concentrations of TCA3

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 ferriprotoporphyrin 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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