



In vitro anti-plasmodial and cytotoxic activities of plants used as antimalarial agents in the southwest Nigerian ethnomedicine

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Abstract

Objective: In order to evaluate the ethnomedical uses of plants as remedy for malaria, in Southwestern Nigeria, six crude methanol extracts obtained from five plant species, identified and selected from ethnomedicine, were studied for *in vitro* anti-plasmodial activity and cytotoxicity. **Method:** The anti-plasmodial properties were evaluated *in vitro*, using the lactate dehydrogenase assay against *Plasmodium falciparum* (multi drug resistant K1) and the cytotoxicity activities were assessed using KB nasopharyngeal cell line. Thereafter, three of the crude extracts were fractionated and subjected to activity studies. **Results:** Methanol extracts of three of the plant extracts; *Cassia siamea* stem bark, *Tithonia diversifolia* leaf and *Cajanus cajan* leaf, were found to display intrinsic anti-plasmodial properties with IC₅₀ values of 24.9 µg/ml, 52.9 µg/ml and 53.5 µg/ml, respectively. Fractionation of three crude extracts using organic solvents led to acquisition of twelve fractions, of which the ethyl acetate fraction of *C. cajan* displayed the highest activity with IC₅₀ value of 15.6 µg/ml. The other active fractions were the aqueous methanol of *C. siamea* bark and ethyl acetate fractions of *Gossypium arboreum*, both displaying anti-plasmodial activities, with an IC₅₀ value of 31.3 µg/ml. The crude methanol extract of *T. diversifolia* was found to be most toxic in the cytotoxicity assay, with an ED₅₀ value of 3.6 µg/ml and least selective to the malaria parasites. **Conclusions:** The ethnomedicine of southwestern Nigeria could provide leads for the discovery of antimalarial drugs.

Key words: Anti-plasmodial activity, cytotoxicity, Nigerian ethnomedicine.

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1. Introduction

Malaria, one of the diseases caused by protozoa is responsible for the high rate of morbidity and mortality in the developing world, especially in the tropical countries. It is estimated that malaria is the cause of the death of between 1.5 and 2.7 million people each year mainly due to an increase in parasite resistance [1, 2].

The therapeutic potential of plants used traditionally as antimalarial remedies cannot be over-emphasized [3, 4] and the effective utilization of existing tools and development of new strategies are critical in the attainment of significant reduction in global malaria mortality by the end of the 1st decade of the 21st century [5].

The search for new antimalarial drug is essential and requires identification of new biochemical targets for drug development and new chemical entities, the most recent example is artemisinin (and its derivatives), isolated from *Artemisia annua* [6, 7].

In a previous study, the results of ethnographic study in 2 communities in Oyo State, Southwestern Nigeria, in addition to the cultural categorization of febrile illnesses in correlation with herbal remedies used for treatment in the region were presented [8]. Further to the social science based study in SW Nigeria, we report herein, the *in vitro* antiplasmodial and cytotoxicity properties of 6 crude methanol extracts, from 6 plant materials obtained from 5 species of plants identified and selected from the earlier study.

After fractionation of three crude extracts to give a total of 12 fractions, their *in vitro* antiplasmodial activities were evaluated. This was done in an attempt to furnish antimalarial chemotherapeutic agents from new plant sources in our continuing studies of the Nigerian antimalarial phytomedicine.

2. Materials and Methods

2.1 Plant collection and authentication

The six plants materials from five plant species evaluated in this study were collected from Otu in Oyo state, Nigeria, in January 2002. All plant samples were authenticated at the Department of Botany, University of Ibadan and Forestry Research Institute of Nigeria (FRIN), Ibadan, where voucher specimens are deposited.

2.2 Plant extraction and fractionation

The materials from the plant species were air-dried and pulverized into powder forms. Material from each species (200g respectively) was extracted into re-distilled methanol (2.5 l) by maceration at room temperature (29°C). Yields of respective extracts were determined (Table 1) and the extracts were stored in the refrigerator until analysis. Fractionation of 3 crude methanol extracts (5.0 g each) were done to give hexane, dichloromethane, ethyl acetate and aqueous methanol fractions of each of the 3 extracts, making a total of 12 fractions and yields of fractions (in g) were determined.

2.3 Parasite strain / *in vitro* culture

The asexual stages of *Plasmodium falciparum* (multi-drug resistant strain K1) obtained from Dr. D.C. Warhurst, London School of Hygiene and Tropical Medicine were cultured continuously according to the modified candle jar method Fairlamb *et al.* [9]

2.4 Parasite Lactate Dehydrogenase (pLDH) assay

The method of Markler and co-workers [10] was used in the estimation of parasite growth inhibition. Cultures were cryopreserved to contain at least 5 % ring form parasites and were maintained at 2-4 % in sterile culture flask with filter caps and complete human A+ washed erythrocytes at 5 % hematocrit, this was used in preparing 2 % hematocrit by washing with phosphate buffered solution (PBS), 3 times. Stock

solutions of extracts were prepared by dissolving known quantities of dried extracts (500 µg) in 1:1 dimethyl sulphoxide (250 µl) and distilled water (250 µl).

Serial dilutions of the extracts/fractions were made in quadruplicates in 96-well microtitre plates. Each well contained 50 µl of the diluted extracts, 50 µl of the complete medium and 50 µl of infected blood. The extract concentrations tested ranged from 500 – 0.5 µg/ml (10 dilutions). Rows of blank made up of water/dimethyl sulphoxide (DMSO), ratio 1:1 and standard antimalarial drug (chloroquine diphosphate, obtained from Sigma Chemical Co, UK) were included in the drug plate.

The drug plate was placed in the chamber with a little sterile water in a Petri dish. The was placed in the laminar flow chamber (Envair, UK) was gassed with pre-filtered mixture of 3 % O₂, 4 % CO₂ and 93 % N₂, then swiftly sealed and incubated at 37°C for 48 h.

2.5 Estimation of *P. falciparum* growth inhibition

After the incubation period, 50 µl of 3-acetyl pyridine dinucleotide (APAD) reagent was added to each well, followed by 50 µl of NBS

reagent and finally after removal of air bubbles, the drug plate was incubated at 37°C for 20 min. Then, optical density was measured in a micro plate reader at 550 nm. This was analyzed with a Wallac counter or MRX a micro plate reader using a Microsoft excel program MsX1fit (IDBS, UK).

The IC₅₀ values (50% inhibition) of six crude extracts from the five plant species and 12 fractions thereof, were determined by plotting the percentage inhibition against the log drug concentration in a linear regression analysis within 95% confidence limit. Chloroquine diphosphate was included in the assay as reference drug.

2.6 *In vitro* cytotoxicity against KB cells

This was determined by the method described by Anderson and co-workers [11]. Briefly, the cytotoxicity assays were carried out in triplicate against human nasopharyngeal carcinoma KB cell lines. After 72 h incubation at 37°C, cell growth was estimated by colorimetric measurement of stained living cells with eosin. The cultured cells were treated at 10 concentrations of the extracts ranging from 1000 - 0.1 µg/mL, and measuring the optical density at 490 nm. Results are presented as ED₅₀ values.

Table 1
Antimalarial Plants from SW Nigerian and yields of crude methanol extracts

| Plant / Authority | Family | Voucher specimen number | Part | Percentage yield ^a (Wts in g) |
|-------------------------------------|-------------|-------------------------|------------|------------------------------------------|
| <i>Cassia siamea</i> Lam | Fabaceae | FHI 106558 | stem bark | 11.5 (23.0) |
| <i>Tithonia diversifolia</i> A.Gray | Asteraceae | FHI 106559 | leaf | 8.9 (17.8) |
| <i>Cajanus cajan</i> Millsp | Fabaceae | FHI 106560 | leaf | 4.3 (8.6) |
| <i>Cassia siamea</i> Lam | Fabaceae | FHI 106558 | leaf | 3.7 (7.4) |
| <i>Gossypium arboreum</i> Linn | Malvaceae | FHI 106561 | leaf | 10.7 (21.4) |
| <i>Lippia multiflora</i> Moldence | Verbenaceae | FHI 106562 | whole herb | 9.8 (19.6) |

a. Yield of extract per 200 g of plant material

Table 2

In vitro Antiplasmodial against K1 strain *P. falciparum* and KB cell Cytotoxicity activities of the methanol plant extracts^{a, b}

| Plant Species | IC ₅₀ K1 in µg/ml | ED ₅₀ KB cells in µg/ml ± SEM |
|------------------------------|---------------------------------|---------------------------------------------|
| <i>Cassia siamea</i> (sb) | 24.9 | 13.3±3.61 |
| <i>Tithonia diversifolia</i> | 52.9 | 3.61±0.59 |
| <i>Cajanus cajan</i> | 53.5 | 28.5±8.50 |
| <i>Cassia siamea</i> (l) | 124.0 | > 300 |
| <i>Gossypium arboreum</i> | 197.9 | > 300 |
| <i>Lippia multiflora</i> | 210.4 | 26.5 ± 6.01 |
| Chloroquine diphosphate | 0.21 | 22.7 ± 2.33 |

a. Each datum represents the mean of two independent experiments (n=10, for SEM).

b. DMSO/H₂O, ratio 1:1 was used for solubilising the extracts and as negative control.

3. Results

The 6 plant materials that were evaluated in this study, voucher numbers of plant species, their plant families and percentage yields of extracts are listed in Table 1. The *in vitro* antiplasmodial study of all 6 extracts, using the multi strain resistant *P. falciparum*, the result, expressed as IC₅₀ values, the result of the cytotoxicity (KB cells, ED₅₀) assay and selectivity indices (ED₅₀ / IC₅₀) of the extracts are listed in Table 2.

In our laboratories, crude extracts and fractions are considered active in the *in vitro* antiplasmodial studies if IC₅₀ values are less than 100µg/mL. Thus, in the present antiplasmodial assay, the stem bark extract of *C. siamea* possessed the highest activity with an IC₅₀ of 24.9µg/mL while the extract of *L. multiflora* (non fruiting shrub) had the lowest activity with IC₅₀ of 210.4µg/mL.

The *in vitro* cytotoxicity assay of the 6 plant crude extracts using KB cells indicated that

T. diversifolia was toxic with an ED₅₀ value of 3.6µg/mL. *Cassia siamea* leaves and *G. arboreum* were the least toxic extracts as shown in Table 2. The results of the 12 fractions of three of the bioactive plant extracts in the *in vitro* antiplasmodial assay and yields of fractions, namely; *Cassia siamea* (bark), *Cajanus cajan* and *Gossypium arboreum* as shown in Table 3.

The ethyl acetate fraction of *C. cajan* exhibited the highest inhibitory activity of the 3 plants (IC₅₀=15.6µg/mL). The dichloromethane (DCM) fraction of *C. cajan*, ethyl acetate fraction of *G. arboreum* and aqueous methanol fraction of *C. siamea* had IC₅₀ values of 31.3µg/mL. The DCM and hexane fractions of *C. siamea* and *G. arboreum* were the least active, IC₅₀=250.0 µg/mL.

4. Discussion and conclusion

Previously, the aerial crude extract of *T. diversifolia* had been evaluated for *in vitro* antiplasmodial activity [12]. Artemesinic acid derivative, sesquiterpenoids as well as sesquiterpene lactones have also been isolated from the extract of the leaf [13] and these may be responsible for the observed antiplasmodial properties

The antiplasmodial properties of *C. cajan* has hitherto not been reported. From the leaf extract of *Cajanus cajan*, Ciranin A, a prenyl stilbene derivative was reported to have displayed antifungal properties [14]. Other compounds previously isolated from the extracts were Cajanol and some benzopyran ketones [15].

Of the *Cassia* species only *C. occidentalis* had been reported to show antiplasmodial properties in addition to our recent studies on the antiplasmodial properties of *C. nigracans* [16, 17]. Anthraquinone derivatives have been isolated from *Cassia* sp., these include

Table 3

Yield (g) and *in vitro* Antiplasmodial against K1 strain *P. falciparum* activities of Plant Fractions ^{a, b, c}

| Fractions | Plant species/IC ₅₀ K1 in µg/ml (yield in g) | | |
|------------------|---------------------------------------------------------|-----------------------|--------------------|
| | <i>C. cajan</i> | <i>C. siamea</i> (sb) | <i>G. arboreum</i> |
| Hexane | 62.5 (1.4) | 125.0 (0.6) | 250.0 (1.2) |
| Dichloromethane | 31.3 (0.9) | 250.0 (1.1) | 125.0 (1.2) |
| Ethyl acetate | 15.6 (1.3) | 62.5 (1.5) | 31.3 (1.3) |
| Aqueous methanol | 31.3 (1.5) | 31.3 (1.6) | 62.5 (0.8) |

a. Each datum represents the mean of two independent experiments (n=10, for SEM).

b. DMSO/H₂O, ratio 1:1 was used for solubilising the extracts and as negative control.

c. Yield is based on 5.0 g of crude methanol extract respectively

chrysopanol and derivatives and bianthraquinones. Other compounds reported from *C. siamea* are benzopyran-8-ones and benzopyran-4-ones [18-20]. *Cassia spp* could be a source of antimalarial compounds.

This is the first report of the antiplasmodial properties of *G. arboreum* leaves. Seeds of the cotton plant, *Gossypium spp*, are known to contain gossypol display both anticancer and antifertility properties [21, 22], this creates the need to investigate the antimalarial compounds in *G. arboreum* especially as the ethyl acetate fractions in the present study had intrinsic antiplasmodial activity, with IC₅₀ of 31.3µg/mL even when the parent crude extract did not display impressive activity.

Currently, no data is available regarding the antimalarial activity of the plant materials, except *T. diversifolia*, neither is there any information on the selectivity of these plant extracts, using KB cells toxicity of these antimalarial plants. *C. siamea* bark and *C. cajan* extracts displayed selective toxicity to malaria parasites with an index of 0.53 each, unlike *T. diversifolia* which was 80 fold, more toxic.

In conclusion, this study has identified a number of plant extracts/fractions, mainly from the Fabaceae plant family (Table 1), that have displayed *in vitro* antiplasmodial activities, based on identification and selection of plants used in the ethnomedicine in SW Nigeria. This is indicative of the contribution of ethnomedicine to the possible discovery of antimalarial drugs.

The ethyl acetate fraction of *C. cajan* leaf was active and the methanol extract was selectively toxic to malaria parasites. The study is continuing in the isolation of the active principles from the fractions that exhibited maximum selectivity to *P. falciparum* in the assay. In addition, toxicological evaluations of active plant compounds in animal models are being assessed.

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