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Original Research Article

In vitro antiplasmodial, cytotoxic and antioxidant effects, and phytochemical constituents of eleven plants used in the traditional treatment of malaria in Akwa Ibom State, Nigeria

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Abstract

Purpose: *To evaluate the antiplasmodial effects of eleven plants (Bombax buonopozense, Carica papaya, Anthocleista djalonensis, Milicia excelsa, Heterotis rotundifolia, Homalim letestui, Starchystarpheta cayennnensis, Ocimum gratissimum, Cleistopholis patens, Chromolaena odorata and Hippocratea africana) reportedly used in the treatment of malaria in Akwa Ibom State of Nigeria.*
Methods: *Phytochemical analysis was done by standard methods, while in vitro antiplasmodial evaluation was carried out using Plasmodium falciparum chloroquine-sensitive and chloroquine-resistant strains using lactate dehydrogenase (pLDH) assay. Cytotoxicity test was undertaken by MTT assay on LLC-MK2 cells and the concentration killing 50 % of the cells (CC_{50}) was calculated. Antioxidant activity of the ethanol extract was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.*
Results: *Milicia excelsa, Heterotis rotundifolia and Chromolaena odorata had moderate antiplasmodial activity. Ocimum gratissimum and Hippocratea africana were weakly active. Milicia excelsa showed a considerable level of cytotoxicity, while Bombax buonopozense exhibited moderate cytotoxicity. Bombax buonopozense (95.3 %) and Ocimum gratissimum (92.0 %) exhibited high DPPH scavenging effect comparable to Vitamin C (98.7 %). There was a significant correlation ($p < 0.05$) between DPPH inhibition and the total phenolic contents of the eleven plants studied ($r^2 = 0.6616$), between DPPH inhibition and flavonoids ($r^2 = 0.3553$), between antiplasmodial activity and saponin content ($r^2 = 0.3992$), and between the two antiplasmodial evaluation assay methods ($r^2 = 0.614$).*
Conclusion: *The results of this work provide some justification for the use of Milicia excelsa, Heterotis rotundifolia, Chromolaena odorata, Ocimum gratissimum and Hippocratea africana in the treatment of malaria.*

Keywords: *Antiplasmodial, Antioxidant, Cytotoxicity, Phytochemicals, Antimalaria*

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INTRODUCTION

World estimate of infections and mortality from malaria annually is 500 million and 2.7 million, respectively. Ninety percent of these infections occur in Africa. Malaria causes about 3000 deaths daily and reduces economic growth by 1.3% per annum in endemic areas [1]. Malaria is the commonest disease in Nigeria, and accounts for a quarter of all cases of the disease in Africa [2]. The spread of *Plasmodium falciparum* resistance to antimalarial drugs has hampered malaria control program. Resistance to Artesunate-Amodiaquine combination therapy, the popular choice for malaria treatment in Nigeria and parts of Africa, has been reported [3]. This development has made the search for novel antimalaria drugs a necessity.

Plants are used as medicine by about 60 % of the world's population [4]. Only 10% of the 250,000 species of plants in the world, have been screened for biological activity [4]. Many Nigerians use plants as remedies against malaria. *In vitro* antiplasmodial and/or *in vivo* antimalarial effects have been demonstrated in the extracts of 45 out of 51 plant species tested. The major proportion of malaria mortality occur in sub-Saharan African regions, it is necessary to encourage studies on plants from these regions.

This study was therefore undertaken to evaluate the antiplasmodial potential of eleven plants used in Akwa Ibom state, Nigeria, for the treatment of malaria. Evaluation of the antioxidant activity of the plants and quantification of some of their phytochemical components were also undertaken. Correlation among the evaluated parameters was determined.

EXPERIMENTAL

Plant collection and extraction

The plants were collected from Uyo metropolis, Akwa Ibom State, Nigeria, in June 2016. They were identified by Professor Magaret Bassey of the department of Botany and Ecological Studies, University of Uyo. The voucher number was assigned to each of the plants as shown in Table 1 and voucher specimens deposited in the herbarium of the Department of Pharmacognosy and Natural Medicine, University of Uyo, Nigeria. The plant materials were air dried and pulverized. Each of the powdered plant material (200 g) was macerated with 70 % ethanol (2 L) for 72 h. The extracts were filtered and concentrated using a rotary evaporator and dried in a desiccator with silica gel.

Quantification of phytochemicals

Determination of Alkaloids was performed according to the method of Harborne [5]. Estimation of saponins was done according to the method of Obdoni and Ochuko [6]. Total phenolic content was determined following the method of Singleton and Rossi [7]. Flavonoid content was evaluated by the method of Ahn *et al* [8]. The quantitative content of carotenoids and lycopene in the leafy extract is determined using the colorimetric method described by Nagata and Yamashita [9].

In vitro antiplasmodial effect

Stock solutions were prepared by dissolving 2 mg dry crude extracts in 200 µL dimethyl sulfoxide (DMSO) from Sigma (MO, USA) and then diluting with complete culture medium to make 2000 µg/mL. All solutions were sterilized by passing through 0.22 µm syringe-adapted filters (Corning®, NY, USA) and stored at 4°C until use. *Plasmodium falciparum* Chloroquine-sensitive (CS2) and *Plasmodium falciparum* Chloroquine-resistant (W2mef) strains were cultured *in vitro* [10] with modifications. Parasites were grown in uninfected O+ human red blood cells as host cells and maintained in complete malaria culture medium composed of RPMI-1640 medium supplemented with $NaHCO_3$ (2 mg/mL), hypoxanthine (10 µg/mL), glucose (2 mg/mL), albumax II (1 %) and gentamicin (10 µg/mL). The parasite cultures were incubated in CO_2 (5 %), O_2 (5 %) and N_2 (90 %) at 37 °C. All the solutions were sterilized with 0.22 µm syringe-adapted filters (Corning®, NY, USA). *In vitro* antiplasmodial activity of the extracts were evaluated using parasite lactate dehydrogenase (pLDH) assay.

Different concentrations of extracts were incubated with non-synchronized 1% parasitized red blood cells (pRBCs) at 2% hematocrit (hct) in 96 well microtiter plates (Costar®, Corning, NY, USA) . Quinine was used as positive control. The test was performed in triplicate for each concentration. Wells with only 1% pRBCs at 2% HCT without extract, were included as negative controls (100 % parasite growth). Wells without pRBCs but with red blood cells only at 2 % HCT served as blank controls. Parasites cultures with extracts were maintained for 48 h at 37 °C in CO_2 (5 %), O_2 (5 %), and N_2 (90 %). After 48 h of incubation, the plates were frozen overnight at -20 ºC and antiplasmodial activity was evaluated using pLDH assay performed as described previously. The concentration of the extracts that inhibited fifty percent of the parasite growth (IC_{50})

was determined by GraphPad Prism, version 7.03

In vitro cytotoxicity test

Stock solutions were prepared by dissolving 1 mg dry crude extracts in 200 µL DMSO (Sigma MO, USA) and then diluting with cell culture medium to make 100 µg/mL. All the solutions were sterilized by using 0.22 µm syringe-adapted filters (Corning®, NY, USA) and kept at 4 °C until use. Cytotoxicity was determined on LLC-MK2 monkey kidney epithelial cells. The cells were grown in DMEM culture medium which was supplemented with 10 % fecal bovine serum (FBS, Life Technologies) and 1 % penicillin/Streptomycin. Trypsinated cells were distributed in 96 well microtiter plates at a density of 10,000 cells/well in a volume of 100 µL per well and incubated for 48 h before adding the extracts. After 48 h, the medium was removed completely from each well, and 100 µL of fresh culture medium was then added. Thereafter, 100 µL of crude extract (2000 µg/mL) was added in row H and then serially diluted to row B to give concentrations ranging from 1000 – 15.6 µg/mL. Cells in row A served as controls without drug (100 % growth). The cells with or without extracts were incubated in CO_2 (5 %), O_2 (5 %), N_2 (90 %) incubator at 37 °C for 72 h before determining their viability. Each concentration was determined in triplicate. Cell viability was determined using MTT assay and the cytotoxic activity was determined according to the previous studies [11]. The percentage viability and percentage mortality were calculated from the absorbance values using Microsoft Excel. The mean results of the percentage mortality were plotted against the logarithms of concentrations using HN-NonLin V1.1 (2002) and GraphPath Prism software. Regression equations obtained from the graphs were used to calculate cytotoxic concentration fifty (CC_{50}), which is the concentration that killed 50 % of the cells.

Evaluation of DPPH-radical scavenging activity

The DPPH antioxidant capacity of the extracts, and vitamin C were evaluated by the method of Enujiugha [12]. A solution of DPPH (0.1 mm) was prepared in methanol and 0.5 ml of this solution added to 1.5 ml of test sample in ethanol at different concentrations (50 - 250 mg/ml). The solutions were vortexed thoroughly and incubated in the dark for about 30 minutes. The absorbance was measured at 517 nm against blank samples. Inhibition (H) of free radical DPPH was calculated as in Eq 1.

$$H (\%) = \{(Ab – As)/Ab\}100 \quad \ldots\ldots\ldots (1)$$

where Ab and As are the absorbance of blank and test samples, respectively.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) with the aid of GraphPad Prism software, version 7.03 for Windows, GraphPad Software, San Diego, California USA). Values of $p < 0.05$ were considered significant.

RESULTS

The names, voucher numbers, and parts of the plants studied are shown in Table 1.

In vitro antiplasmodial property

The antiplasmodial property of the extracts on two reference laboratory strains of *P. falciparum* is presented in Table 2. Considering significant activity in terms of IC_{50} below 10 on at least two strains, five out of the eleven tested extracts can be classified as having promising activity.

Table 1: Plants studied and their voucher numbers

Plant name (family)	Part	Voucher no.
Anthocleista djalonensis (Gentianaceae)	Roots	UUPH45a
Bombax buonopozense (Malvaceae)	Leaves	UUPH31a
Carica papaya (Caricaceae)	Leaves	UUPH18a
Chromolaena odorata (Asteraceae)	leaves	UUPH10c
Cleistopholis patens (Annonaceae)	Root	UUPH4f
Heterotis rotundifolia (Melastomataceae)	leaves	UUPH48a
Hippocratea africana (Celastraceae)	Root	UUPH34a
Homalim letestui (Flacourtiaceae)	Stem bark	UUPHA69i
Milicia excelsa (Moraceae)	Stem bark	UUPH50b
Ocimum gratissimum (Labiatea)	leaves	UUPH38a
Starchystarpheta cayennnensis (Verbenaceae)	Leaf	UUPH78c

Table 2: Antiplasmodial activity (IC_{50}) of the extracts on chloroquine-sensitive *Plasmodium falciparum* (CS2) and *Plasmodium falciparum* Chloroquine-resistant (W2mef) strains

Plant	IC_{50} on CS2	IC_{50} on W2 (µg/mL)	Observation
Anthocleista djalonensis	3.55 ± 0.95	>1000	Inactive
Bombax buonopozense	2.94 ±0.03	125.23 ± 8.23	Inactive
Carica papaya	>1000	>1000	Inactive
Chromolaena odorata	3.89 ± 0.92	7.12 ± 4.88	Moderately active
Cleistopholis patens	21.43 ± 2.45	14.68 ± 1.02	Inactive
Heterotis rotundifolia	4.63 ± 0.02	7.80 ± 0.73	Moderately active
Hippocratea africana	15.66 ± 2.92	6.95 ± 0.09	Weakly active
Homalim letestui	>1000	222.20 ± 2.24	Inactive
Milicia excelsa	6.43 ± 1.27	3.88 ± 2.49	Moderately active
Ocimum gratissimum	23.23 ± 3.35	4.17 ± 0.23	Weakly active
Starchystarpheta cayennnensis	>1000	>1000	Inactive
Quinine	0.09 ± 0.005	0.12.50 ± 0.03	

IC_{50} < 0.03 : Highly active ; 0.03 < IC_{50} < 2.5: active ; 2.5 < IC_{50} < 10 : moderately - weakly active; IC_{50} > 10 : inactive [15]. Mean and SEM values were generated from three replicates of each assay

Cytotoxicity profiles of the extracts

Table 3 presents the cytotoxic concentration 50% (CC_{50}) for the extracts on the LLC-MK2 Monkey kidney epithelial cell line. From this data, two out of the 11 extracts exhibited moderate toxicity against LLC-MK2 cell line, namely *Milicia excelsa* and *Bombax buonopozense*, with CC_{50} between 10 and 30 µg/mL.

Antioxidant activity of the extracts

Results of DPPH scavenging property of the plant extracts are shown in Figure 1.

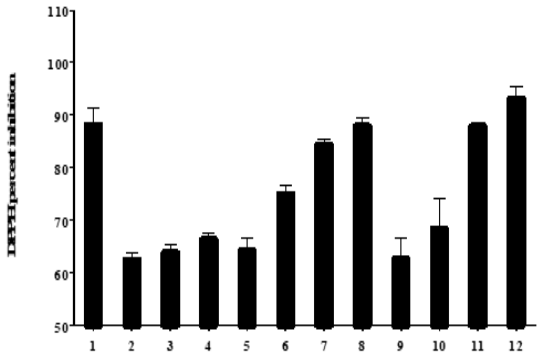

Figure 1: DPPH percent inhibition of the plants. 1. *Bombax buonopozense, 2. Carica papaya, 3. Anthocleista djalonensis, 4. Milicia excelsa, 5. Heterotis rotundifolia, 6. Homalim letestui, 7. Starchystarpheta cayennnensis, 8. Ocimum gratissimum, 9. Cleistopholis patens, 10. Chromolaena odorata, 11. Hippocratea africana, 12. Vitamin C*

Phytochemical profile of the extracts

The composition of phytochemical constituents of the plants are as shown in Table 4.

The results of the correlation analysis are shown in Table 5

Table 3: Cytotoxicity (CC_{50}) of the plant extracts

Plant	CC_{50} on LLC-MK2 (µg/mL)	Observations
Anthocleista djalonensis	316.83 ± 1.70	Non-cytotoxic
Bombax buonopozense	22.12 ± 6.38	Moderately cytotoxic
Carica papaya	170.54 ± 6.96	Non-cytotoxic
Chromolaena odorata	35.17 ± 3.79	Non-cytotoxic
Cleistopholis patens	415.63 ± 6.82	Non-cytotoxic
Heterotis rotundifolia	542.63 ± 15.45	Non-cytotoxic
Hippocratea africana	48.60 ± 4.11	Non-cytotoxic
Homalim letestui	52.41 ± 10.15	Non-cytotoxic
Milicia excelsa	10.95 ± 0.55	Cytotoxic
Ocimum gratissimum	486.38 ± 4.57	Non-cytotoxic
Starchystarpheta cayennnensis	438.97 ± 13.20	Non-cytotoxic
Gleevec (Imatinib)		Weakly cytotoxic

CC_{50}, = Cytotoxic concentration 50 %. CC_{50} < 5: highly toxic; 5 < CC_{50} < 10: cytotoxic; 10 < CC_{50} < 30: Moderately to weakly cytotoxic; CC_{50} > 30: Non-cytotoxic [14]. Mean and SEM values were generated from three replicates of each assay

Table 4: Phytochemical composition of the plants

Plant	Phenolics (mg/mL)	Flavonoids (mg/mL)	β-Carotene (mg/100 mL)	Lycopene (mg/100 mL)	Saponins (% w/w)	Alkaloids (% w/w)
Anthocleista djalonensis	0.0508±0.01	0.0031±0.000	0.0436±0.00	0.0129±0.002	0.26±0.01	0.44±0.01
Bombax buonopozense	0.2912±0.03	0.0058±0.000	0.4199±0.01	0.0895±0.005	0.10±0.01	0.32±0.01
Carica papaya	0.0881±0.01	0.0309±0.004	0.8220±0.03	0.1045±0.006	0.94±0.01	4.98±0.08
Chromolaena odorata	0.0858±0.01	0.0198±0.004	7.4955±0.09	0.8723±0.007	0.32±0.01	3.32±0.05
Cleistopholis patens	0.1715±0.01	0.0115±0.001	0.4666±0.03	0.0893±0.002	0.44±0.01	3.86±0.03
Heterotis rotundifolia	0.1611±0.02	0.0152±0.002	2.9937±0.06	0.1978±0.006	0.22±0.01	2.48±0.02
Hippocratea africana	0.3973±0.02	0.1938±0.006	0.3721±0.03	0.0094±0.000	0.84±0.01	2.42±0.02
Homalim letestui	0.1662±0.03	0.0103±0.001	0.0343±0.00	0.0063±0.000	0.32±0.01	0.42±0.01
Milicia excelsa	0.1270±0.03	0.0109±0.001	0.0436±0.00	0.0092±0.000	0.24±0.01	1.38±0.03
Ocimum gratissimum	0.6800±0.03	0.0796±0.001	0.9096±0.04	0.0563±0.002	0.44±0.01	4.72±0.09
Starchystarpheta cayennnensis	0.3666±0.03	0.0675±0.002	0.6779±0.01	0.0522±0.004	1.28±0.07	3.26±0.03

Table 5: Correlation (r^2) between phytochemicals and activities (antiplasmodial, cytotoxicity and antioxidants)

Activity	Phenolics	Flavonoids	β-Carotene (mg/100 ml)	Lycopene (mg/100 ml)	Saponins (%)	Alkaloids (%)
Antiplasmodial	0.004119	0.008835	0.07506	0.07574	0.3992*	0.03498
cytotoxicity	0.1087	0.01321	0.06315	0.09560	0.04415	0.2226
DPPH inhibitory activity	0.6616*	0.3553*	0.004784	0.01254	0.05372	0.007468

*P < 0.05 (two-tailed)

DISCUSSION

Stembark extract and root of *Milicia excelsa* are used to treat fever and malaria in the southern part of Nigeria [15]. The result of this study showed that *Milicia excelsa* possessed *in vitro* antiplasmodial activity, thereby justifying the ethnobotanical use of the plant extract for the treatment of malaria. The results of this study also showed that the stem bark of the plant is cytotoxic (CC_{50} on LLC-MK2 is 10.95 ± 0.55 µg/mL). The root extract of the plant has earlier been reported to be cytotoxic. This plant should therefore be used with caution because of its cytotoxicity.

The leaf of *Carica papaya* is reportedly used in ethnomedicine for malaria treatment, but the result of this study does not confirm this claim.

The methanol leaf extract of *B. buonopozense* has been reported to possesses significant antiplasmodial activity thus confirming its traditional use in malarial therapy [16]. But the ethanol leaf extract used in this study did not show any antiplasmodial activity. However, the antiplasmodial property of plant extracts has been reported to vary with the solvent used for extraction and this may account for the differences between the reported antiplasmodial property of the plant and the results obtained in this study.

Anthocleista djalonensis leaf extract reportedly exhibited a significant antiplasmodial activity [17]. However, in this study the root extract was found to be inactive. This shows that, unlike the leaf and stem bark, the root extract of the plant may not have any beneficial antiplasmodial activity. This study shows that the ethanol leaf extract of *Heterotis rotundifolia* plant possesses moderate antiplasmodial activity. There is scanty information in literature on the antiplasmodial activity of this plant.

From the results of this study, the stem bark of *Homalim letestui,* did not possess antiplasmodial activity. This result is in tandem with earlier report [18]. However, *in vivo* study reported the significant antiplasmodial activity of the root [23]. Therefore, the root, rather than the stem bark and leaf, of the plant could be exploited further for malaria treatment.

The results of this study showed of *Stachytarpheta cayennensis* leaves do not possess antiplasmodial activity. This result is at variance with the report that the leaf extract exhibited significant blood schizonticidal property comparable to chloroquine [19].

In this study ethanol extract of the leaf of *Ocimum gratissimum* possessed weak antiplasmodial, activity. This result agrees with the report of the antiplasmodial activity of the leaves on *P. falciparum* F32. The results of this work showed that the root extract of *Cleistopholis*

patens possesses no antiplasmodial effect and is non-cytotoxic. However, the stem bark is reportedly used in the treatment of malaria in Cameroon [20].

Leaf extract of *Chromolaena odorata* according to this study show moderate antiplasmodial activity. This agrees with the reported antiplasmodial activity of the plant [21]. Results of the antiplasmodial activity of the root extract of *Hippocratea africana* show that the root extract is moderately active, in agreement with the reported activity of the ethanol root extract of the plant.

There was a significant correlation ($r^2 = 0.6616$, $p < 0.05$) between the DPPH percent inhibition and total phenolic contents of the eleven plant extracts tested in this study. The correlation between DPPH inhibitory activity and flavonoids was also significant ($r^2 = 0.3553$, $p < 0.05$). This is expected since phenolics in general and flavonoids in particular are well known to be responsible for the antioxidant properties of plants.

Similarly, the correlation between antiplasmodial activity and saponin content of the plant extracts was significant ($r^2 = 0.3992$, $p < 0.05$). This result show that the antiplasmodial components of these plants may be reasonably attributable to their saponin contents. Many saponins have been known to exhibit antiplasmodial properties. There was no significant correlation between the antiplasmodial and antioxidant effects of the plants. The two antiplasmodial evaluation assays using *Plasmodium falciparum* Chloroquine-sensitive (CS2) and *Plasmodium falciparum* Chloroquine-resistant (W2mef) strains) had a significant correlation of 0.614.

CONCLUSION

The results of this study provide some support for the use of *Milicia excelsa, Heterotis rotundifolia*, *Chromolaena odorata*, *Ocimum gratissimum* and *Hippocratea africana* in malaria treatment. *Milicia excelsa* and *Bombax buonopozense* should be used with caution because of their cytotoxicity. *Bombax buonopozense* and *Ocimum gratissimum* show high DPPH inhibitory activity comparable to that of vitamin C.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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