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ANTIMALARIAL ACTIVITY AND CYTOTOXICITY PROFILE OF THE HEXANE, DICHLOROMETHANE AND METHANOL SEED EXTRACTS OF *AFRAMOMUM DANIELLI* K. SCHUM (ZINGIBERACEAE)

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ABSTRACT

Introduction: The search for alternatives to the artemisinins (the bedrock of modern antimalarial chemotherapy) is a resultant response to the emergence of the development parasite resistance to the artemisinins. **Methods**: This study evaluated the *in vitro* and *in vivo* antimalarial activity and the cytotoxicity profile of the *n*- hexane, dichloromethane and methanol extracts of *Aframomum danielli* seeds. *In vitro* susceptibility of chloroquine-sensitive *P. falciparum* D10 strain to the extracts was evaluated using parasite lactate dehydrogenase assay, while cytotoxicity was determined using 3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazoliumbromide (MTT) assay with Vero cells and emetine as standard drug. Chloroquine-resistant *P.berghei* (ANKA) infected Swiss mice allotted into 14 groups of 5 per group: corn oil (5mL/kg), 50, 100, 200, 400 mg/kg of each extract and chloroquine (10 mg/kg) were used to evaluate *in vivo* antimalarial activity in a 4-day suppressive test. **Results**: The *n*-hexane, dichloromethane and methanol extracts of the seeds of *Garcinia kola* were active *in vitro* against chloroquine sensitive *P. falciparum* D10 strain with IC_{50} values ≤ 16 µg/mL. The hexane and dichloromethane extracts were non- cytotoxic against Vero cells with IC_{50} values ≥ 122 µg/mL. The hexane extract of *Aframomum danielli* seeds reduced parasitemia by 100% at 200 mg/kg and 400 mg/kg with prolonged survival in mice infected with *P.berghei* ANKA. **Conclusion**: The observed antimalarial activity justifies the use of *Aframomum danielli* seeds in the treatment of febrile illnesses.

KEYWORDS: Antimalarial, *Aframomum danielli* seeds, *P. falciparum* D10, Cytotoxicity, Artemisinins, chemosuppression.

INTRODUCTION

The incidence of malaria and mortality attributable to malaria between 2000 and 2015 is estimated to have decreased by 60% and 37% respectively.[1] These resultant dividends are as a result of a combination of an increase in the use of artemisinin-combination therapy, the use of insecticide-treated nets, residual spraying and intermittent preventive treatment. Unfortunately, the emergence and spread of artemisinin resistant *Plasmodium falciparum* in Southeast Asia threatens the gains and the potential prospects for the elimination of the infection.[2,3] In the absence of an effective vaccine in sight, there is an urgent need for the discovery and development of new chemotherapeutic agents that would serve as leads for the synthesis of new antimalarials to meet the challenges of drug resistance in malaria. One possible source for such safe, effective and affordable alternatives may be medicinal plants.

Plant derived compounds have played key roles in drug discovery.[4,5] Quinine and artemisinin (the foundation of modern antimalarial chemotherapy) were obtained from plants. Several studies have been carried out to evaluate the inhibitory effects of various plant extracts on *Plasmodium falciparum and Plasmodium berghei*,[6-10] *Aframomum danielli*, (Hook, F) K. Schum (family, Zingiberaceae) is a medicinal plant with potential antimalarial activity. It is a large, robust perennial plant 3-4m tall which grows in central and West African countries. It is also commonly known as alligator pepper or mbongo spice or hepper pepper. The seeds of this plant are used for flavouring traditional dishes and for dye preparations, with Its' essential oil used in perfumery.[11]

Studies have shown that extracts of *Aframomum danielli* contain phytochemicals like alkaloids, flavonoids, carotenoids and possesses antioxidant, antimicrobial and antiangiogenic properties,[12,13] however, limited information exists on its antimalarial potential.

Efforts in this study were devoted to the evaluation of the *in vitro* and *in vivo* antimalarial potential of the hexane, dichloromethane and methanol seed extracts of *Aframomum danielli* against chloroquine sensitive *P. falciparum* D10 strain and chloroquine-resistant

P.berghei in mice, respectively. The cytotoxicity profile of the hexane, dichloromethane and methanol seed extracts of *Aframomum danielli* against Vero cells was also evaluated.

MATERIALS AND METHODS

Plant Materials

Collection and Preparation of Plant extracts

The seeds of *Aframomum danielli* were air-dried and pulverised to a coarse powder using a table top blender. Five hundred grams (500 g) of the pulverised seeds was extracted exhaustively by cold maceration successively in hexane, dichloromethane and methanol at room temperature for 72 hours. The extracts were filtered, and the filtrates were concentrated to dryness using a rotary evaporator. The extracts were stored in a refrigerator (4°C) until required for assay.

Drugs and chemicals

All reagents/chemicals used in this study were of analytical grade. Sodium acetate and HEPES were purchased from Fluka. Pyridine 99%, glacial acetic acid and dimethylsulfoxide (DMSO; 99.7% purity) were purchased from Acros Organics. Hydrochloric acid 1M and sodium hydroxide 0.1M were purchased from Merck. All other chemicals used in this study were purchased from Sigma-Aldrich®, UK.

Antimalarial activity

In vitro antimalarial activity was determined using the parasite lactate dehydrogenase assay.[14] Chloroquine-sensitive *P. falciparum* D10 strain derived from FCQ-27 from Papua New Guinea donated by the Division of Pharmacology at the University of Cape Town, South Africa was used for this study. The parasites were grown and maintained in continuous culture using a modification of the method of Trager and Jensen.[15] The culture medium consisted of 2% haematocrit suspension of O^+ human erythrocytes in RPMI (Roswell Park Memorial Institute Medium) 1640, supplemented with phenol red, 25mMNaHCO3, 25mMHEPES buffer (pH 7.4), and 50 μg/mL gentamycin and hypoxanthine. The reagents were obtained from Sigma-Aldrich, South Africa.

The plant extracts (2 mg) were initially dissolved in DMSO (200 µL) and then diluted in complete culture media to 200 µg/mL. Two-fold serial dilutions were done with culture medium in a 96-well plate to give an extract concentration range of 0.195-100 µg/mL. One hundred microliter (100uL) of an asynchronous culture of *P. falciparum* at 2% parasiteamia and 2% haematocrit was added to each well of the 96-well plate and incubated for 48 hours at 37 ^oC. After incubation, the parasites were re-suspended by mixing gently using a multi-channel micropipette. Twenty microliter (20 µL) of the culture was taken from each well and added to a new 96-well microtitre plate containing 100 µL of the Malstat reagent in each well. Twenty-five microliter (25 µL) of a solution of 1.9 µM Nitro Blue Tetrazolium (NBT) and 0.24 µM Phenazine Ethosulphate (PES) were added to each well thus initiating the lactate dehydrogenase reaction.

Parasite growth was determined spectrophotometrically at 620nm with the aid of a microplate reader after an hour of incubation in a dark cupboard. Absorbance values obtained were expressed as a percentage of the 100% growth value and plotted against corresponding concentrations of the drug. The IC_{50} values of the plant extracts were obtained from dose-response curves, using non-linear dose-response curve fitting analyses with Graph Pad Prism version 5.0. Extracts with $IC_{50} \leq 10$ µg/mL were described as having good activity, while those with IC_{50} values > 10 µg/mL ≤ 30 µg/mL were described as having a moderate activity. Extracts with IC_{50} values ≥ 30 µg/mL were described as being inactive.

In vivo antimalarial tests

In vivo tests were performed according to the NIH guide for the care and use of laboratory animals, (NIH, 2010), and approved by the University of Ibadan ethical committee for the use of Laboratory animals. Inbred Swiss albino mice, weighing between 20-22g, aged 8-9 weeks, were used for all experiments. Animals were obtained from the animal house of the Malaria Research Laboratories, Institute for Advanced Medical Research and Training (IMRAT), University of Ibadan. The mice were housed in groups of five in plastic cages, fed with mouse cubes and provided with water ad libitum. The Peters' 4-day suppressive test was used for the *in vivo* drug tests.[16]

Parasites

Chloroquine-resistant *Plasmodium berghei* (ANKA) strain used was obtained from the Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan. Parasitized red blood cells were obtained from donor-infected mouse by cardiac puncture in acid citrate dextrose (ACD) anticoagulant.

Experimental animals

Seventy albino mice weighing 18-22 g were inoculated intravenously with 1×10^6 red blood cells infected with the CQ-resistant *P. berghei* ANKA strain. The infected animals were distributed randomly into 14 groups of 5 animals each and were treated with 50, 100, 200 or 400 mg/kg body weight of the hexane, dichloromethane and methanol extracts of ADS for 4 days. Two controls groups were used: one treated with chloroquine 10 mg/kg body weight given daily for 3 days while the second group of animals received corn oil (vehicle solution). Parasitemia was determined by microscopic examination of Giemsa–stained blood films obtained from the tail snips from each animal on day 4 after infection. The number of parasitized erythrocytes among 1000 RBC was counted and the percentage chemosuppression for each extract was calculated.

Animal survival was monitored daily, until 30 days after infection.

1. Group I: Negative Control (received corn oil)
2. Group II: Positive Control (infected mice that received chloroquine 10 mg/kg body weight)
3. Group III-VI: infected mice that received 50,100,200 or 400 mg/kg body weight of the hexane extract of ADS
4. Group VII-X: infected mice that received 50,100,200 or 400 mg/kg body weight of the dichloromethane extract of ADS
5. Group XI-XIV: infected mice that received 50,100,200 or 400 mg/kg body weight of the methanol extract of ADS

Cytotoxicity Screening

The Vero cells obtained from the kidney epithelial cells of the African green monkey were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine, penicillin (100 units/mL) and streptomycin (100 µg/mL). The cells were cultured at 37ºC in a humified 5% CO_2 incubator. The cytotoxicity of the seed extracts of *Aframomum danielli* was evaluated against vero cells using the MTT (3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay as described by Mossman[17] and emetine, was used as a standard cytotoxic agent. Briefly, the vero cells grown in standard tissue culture were plated at a density of 1x 10^6 cells/mL/well (100 µL) into a 96-well plate.

One hundred microliter (100 µL) of complete medium was also dispensed into the 96-well plate and cells were allowed to attach for 24 hours at 37°C. The medium was completely removed after 24 hours and replaced with 100 µL of fresh medium containing graded concentration of 100-0.0001µg/mL of the extracts of *Aframomum danielli* seeds. A blank containing only complete medium served as a control containing no drug. The plates were then incubated at 37°C in 5% $CO_{2\ for}$ 48 hours. Thereafter, 25 µL of sterile MTT (5 mg/mL in PBS) was dispensed into each well and incubated for 4 hours at 37°C. Cell viability was measured at 540nm on a microtiter plate reader. Cell viability in the presence of the extract were compared to that of the negative control. The fifty percent inhibitory concentrations (IC_{50}) were calculated for the extracts and the positive control (emetine) using non-linear regression analysis. *In vitro* selectivity index was determined for each extract as the IC_{50} for Vero cells/ IC_{50} for *P.falciparum.*

Statistical analysis

Graph Pad prism version 5.0 (Graph-Pad Software, San Diego, CA, USA) was used for all statistical analyses. Fifty percent inhibitory concentration (IC_{50}) was calculated using non-linear regression analysis. Chemosuppression of parasite growth and survival of animals were expressed as mean ± SEM. Statistical significance of means of different variables were analysed using Student's t test and one way analysis of variance between groups (ANOVA) was used to compare difference in percentage inhibition of parasite growth. For all statistical tests, P=0.05 was considered significant.

RESULTS

***In vitro* antimalarial assay**

The *in vitro* antimalarial activity of the three seed extracts of *Aframomum danielli* against the D10 strain of *P.falciparum* is presented on Table 1. The hexane extract exhibited the highest *in vitro* antimalarial activity, with an IC_{50} value of 9.04±0.32 µg/mL while the dichloromethane and the methanol extract produced moderate activity with IC_{50} values of 15.36±1.03 and 10.23±0.39 µg/mL against the D10 strain of *P.falciparum* respectively. For the evaluation of the *in vitro* antimalarial potential of plant, seed extract or potential antimalarial agents, lower fifty percent inhibitory concentration values (IC_{50}) are indicative of the ability of the plant, seed extract or potential antimalarial agent to destroy parasites at a lower concentration. Invariably, the hexane extract of *Aframomum danielli* seeds produced the highest IC_{50} value, indicative of its ability to destroy fifty percent of *Plasmodium falciparum* at a concentration of about 9.04±0.32 µg/mL.

Table1: Susceptibility profile of Chloroquine-sensitive *P.falciparum* D10 strain to seed extracts of *Aframomum danielli.*

Name of extract	IC_{50} (µg/mL)
Aframomum danielli hexane	9.40 ±0.32
Aframomum danielli dichloromethane	15.36±1.03
Aframomum danielli methanol	10.23±0.39
Chloroquine	0.12±0.03

In vivo antimalarial assay

The *in vivo* antimalarial activity of the hexane, dichloromethane and methanol extracts of *Aframomum danielli* seeds (ADS) against *P. berghei* ANKA strain in Swiss albino mice is presented on Table 2. Mean Parasitaemia on day 4 in the untreated control animals was 13.43±0.65%, while the parasiteamia in animals treated with selected doses of ADS extracts (50–400 mg/kg) ranged from 0.0% to 31.6%. A dose-dependent chemosuppression of parasite growth was observed in animals treated with the hexane, dichloromethane and methanol extracts of ADS.

The hexane extract of ADS produced the highest chemosuppression of 100% at doses of 200 and 400 mg/kg comparable to the chemosuppression produced by

chloroquine, which also completely suppressed parasiteamia on D4. At 100, 200 and 400 mg/kg of the hexane extract of ADS tested, there was a significant percentage inhibition of parasiteamia compared to the untreated control group. The animals in the group treated with the dichloromethane extract of ADS did not produce significant chemosuppression of parasiteamia, however, the animals survived longer than those in the untreated group. The methanol extract of ADS produced no chemosuppression of parasiteamia at all doses tested but all the animals treated with the selected doses of the methanol extract of ADS survived longer than the animals in the untreated group. Animals in the group treated with 400 mg/kg of the hexane extract of ADS exhibited the longest survival time of 17 days and survived longer than the animals in the untreated group.

Table 2: *In vivo* antimalarial activity of the seed extracts of *Aframomum danielli* against chloroquine resistant *P.berghei* in Swiss albino mice.

Dose mg/kg	Parasiteamia ± SEM (%)	Chemosuppression ± SEM (%)	Survival time (days)
AD HEX			
50	11.10±0.55	17.30±0.60	10.50±0.50
100	5.39±0.20	59.50±1.50	12.80±1.20
200	0.00±0.00	100.00±0.00	13.40±1.40
400	0.00±0.00	100.00±0.00	16.90±2.90
AD DCM			
50	13.32±0.38	0.00±0.00	10.60±0.20
100	12.59±0.66	6.35±0.65	11.50±0.10
200	11.74±0.31	12.70±1.80	12.30±0.30
400	10.83±1.07	19.50±3.90	14.85±0.85
AD MEOH			
50	31.55±2.25	0.00±0.00	10.50±0.50
100	26.93±1.27	0.00±0.00	11.70±0.30
200	21.01±1.23	0.00±0.00	12.60±0.60
400	16.57±1.17	0.00±0.00	14.15±0.15
C/Q 10mg/kg	0.00±0.00	100.00±0.00	31.50±3.50
Corn oil	13.43±0.65	0.00±0.00	10.60±0.20

Cytotoxicity screening

The Cytotoxic activity of the hexane, dichloromethane and methanol extracts of ADS against Vero cells is shown in Table 3. The IC_{50} values of the extracts against vero cells ranged from 15. 21 µg/mL to 144.80 µg/mL. The dichloromethane extract of ADS was the least toxic (IC_{50} = 144.80 ± 3.60 µg/mL) while the methanol extract was the most toxic (IC_{50} = 15.21 ± 1.01µg/mL). The IC_{50} value of emetine, a standard cytotoxic agent which served as the positive control was 8.00 ± 0.60 µg/mL. All the extracts of ADS evaluated displayed selectivity for *P.falciparum* (Selectivity index ranged between 1.49 and 13.57) (Table 3).

Table 3: *In vitro* cytotoxicity profile of the seed extracts of *Aframomum danielli* against Vero cells.

Name of extract	IC_{50} (µg/mL)	Selectivity index
Aframomum danielli hexane	122.70 ±2.40	13.57
Aframomum danielli dichloromethane	144.80±3.06	9.43
Aframomum danielli methanol	15.21±1.01	1.49
Emetine	8.00±0.60	N/A

DISCUSSION

The spread of parasites resistant to the artemisinins,[18,19] characterised by slower parasite clearance rates, has necessitated the search for newer and more potent antimalarial agents that can serve as alternatives. The focus of this study was to explore medicinal plants that could serve as templates for the development of these newer antimalarial agents. *Aframomum danielli* seeds are used indigenously for the treatment of febrile illnesses. Aframomum spp such as *A.melegueta*, *A.zambessiacum* and *A.latifolium* are used indigenously for the treatment of malaria symptoms in West Africa,[6,20] Thus *Aframomum spp* serves as a valuable product to be explored for antimalarial activity.

Results from this study indicate that the hexane, dichloromethane and methanol extracts of ADS possess good to moderate *in vitro* antimalarial activity. The hexane extract of *Aframomum danielli* seeds demonstrated the highest activity *in vivo* against the asexual erythrocytic stages of *P.berghei.* Data from the present study adds to the repository of species of Aframomum with potential antimalarial activity.[3,20] Duker-Eshum and his colleagues investigated *A.latifolium and A.sceptrum* and extracts from both plants exhibited significant *in vitro* activity.[6]

Kenmogne and his colleagues also reported good *in vitro* activity in five diterpenoids isolated from

A.zambesiacum.[20] Despite the fact that all the three extracts of ADS displayed good to moderate in vitro activity, only the hexane extract displayed antimalarial activity *in vivo* against *P.berghei.* The exact reason for this is not fully understood however, various factors such as first pass effect, poor absorption and route of administration of the extracts and fractions may have influenced the activity of the extracts. It is interesting to note that the hexane and dichloromethane extracts of *Aframomum danielli* seeds were found to be non-cytotoxic to normal monkey epithelial cells. This is indicative of the selectivity of these extracts to destroy parasites and as such can be considered as potential leads for development of antimalarial drugs for clinical management of malaria.

CONCLUSION

The observed antimalarial activity of the hexane extract of *Aframomum danielli* seeds and its non-cytotoxic profile seems to justify its use in folklore for the treatment of febrile illnesses, thus further studies on the purification of the hexane extract, its probable mechanism of action and the determination of its chemical constituents is necessary.

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