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PROBABLE MECHANISM OF ACTION AND GAS CHROMATOGRAPHY-MASS SPECTROSCOPY PROFILE OF THE ANTIMALARIAL FRACTIONS OF THE HEXANE SEED EXTRACT OF *AFRAMOMUM DANIELLI* K. SCHUM (ZINGIBERACEAE)

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ABSTRACT

Introduction: There is an urgent need for the discovery of new chemotherapeutic agents that could serve as alternative drugs or leads for the synthesis of new antimalarials. One possible source for that might lie in ethno-medicine. **Methods**: This study evaluated the antimalarial activity, mechanism of action and constituents of the hexane extract of *Aframomum danielli* seeds and its column-fractions. The *in vivo* antimalarial activity of the hexane extract of AD and its column fractions against Chloroquine-resistant *Plasmodium berghei* (ANKA) strain, the mechanism of action and the constituents of the column fractions was determined using the Peters' 4-day suppressive test, the beta hematin assay and gas chromatography and mass spectrometry respectively. **Results**: In this study, ADHF1 and ADHF7, two of the eight column-fractions obtained from the hexane extract of *Aframomum danielli* seeds and screened for *in vivo* antimalarial activity produced a chemosuppression of 63.9% and 62.4% respectively at 200 mg/kg. Both fractions inhibited the conversion of heme to heamozoin and contained arenes and terpenoids following gas chromatography-mass spectrometry. **Conclusion**: The observed antimalarial activity observed justifies the use of *Aframomum danielli* seeds in the treatment of febrile illnesses.

KEYWORDS: Antimalarial activity, mechanism of action, Aframomum danielli, heme, heamozoin Arenes, terpenoids.

INTRODUCTION

There is an urgent need for the discovery and development of new chemotherapeutic agents that could serve as alternative drugs or leads for the synthesis of new antimalarials as a result of the emergence and spread of resistant parasites to the artemisinins.[1,2] Medicinal plants might be a source of such alternatives based on antecedence of drug discovery especially that of antimalarial agents.[3,4]

Aframomum danielli, (Hook, F) K. Schum (family, Zingiberaceae) is a medicinal plant found in some West African countries. Studies have shown that extracts of *Aframomum danielli* contain phytochemicals like alkaloids, flavonoids, carotenoids and possesses antioxidant, antimicrobial and antiangiogenic properties.[5,6] and more recently, the *in vivo* antimalarial potential of hexane extract of the seeds of *Aframomum danielli* was reported.[7] Limited information however exists on the mechanism of action and the phytochemical constituents of the column fractions of the antimalarial hexane extract of *Aframomum danielli* seeds.

Efforts in this study were devoted to the evaluation of the *in vivo* antimalarial activity of the column- fractions of the hexane extract of *Aframomum danielli* seeds against chloroquine-resistant *P.berghei* in mice, the probable mechanism of action and the phytochemical constituents of the active extract and its column-fractions.

MATERIALS AND METHODS

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.

Preparation of Plant extract

The seeds of *Aframomum danielli* were obtained from Molete market, Ibadan, Nigeria. The proof of authentication was issued by Mr A.A Ekundayo, a plant taxonomist at Forestry Research Institute of Nigeria Ibadan (authentication number FHI 109889).

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 at room temperature for 72 hours. The extract was filtered, and the filtrate was concentrated to dryness using a rotary evaporator. The extract was stored in a refrigerator (4°C) until required for assay.

In vivo Antimalarial Activity

In vivo tests were performed according to the NIH guide for the care and use of laboratory animals, NIH publication (volume 25, number 28), revised 2011). 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.[8]

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. 1. Parasitized red blood cells were obtained from donor- 2. infected mouse by cardiac puncture in acid citrate dextrose (ACD) anticoagulant. 3.

Fractionation of the hexane extract of *Aframomum danielli* seeds

Based on the observed *in vivo* antimalarial activity of the seed extracts of *Aframomum danielli*, the hexane extract of ADS was fractionated on silica gel using column chromatography on silica gel according to the method by Bajpal. The hexane extract of ADS was chromatographed on silica gel with size of 230-400 mesh (Biopharma India) in a glass column (47cm x 3cm). Briefly, the column was packed with 200 g of silica gel using hexane. Fifteen grams (15 g) of the hexane extract was adsorbed with silica gel, dried, packed into the column and chromatographed using a gradient solvent system consisting of hexane and ethyl acetate (100% hexane, 95:5 hexane/ETOAc, 90:10 hexane/ETOAc…...) to elute the column. Seventy-six fractions were obtained and spotted on thin layer chromatographic plates.

According to the method by Bajpal, the plates were developed in a chamber containing the corresponding elution solvent of each fraction. Plates were allowed to dry and thereafter introduced into concentrated sulfuric acid and then into a chamber containing iodine crystals. The plates were then viewed for similarities in retention times. Fractions with similar retention times were pooled based on their TLC profiles. A total of twenty- eight (28) fractions pooled fractions were obtained and stored at 4^{o}C until when needed.

Evaluation of the *in vivo* antimalarial activity of the column-fractions of the hexane extract of *Aframomum danielli* seeds.

The Peters' 4-day suppressive test was used for the *in vivo* antimalarial assay. Based on the percentage yield of the column -fractions, eight (8) hexane column-fractions were evaluated.

Fifty (50) Swiss albino mice inoculated intravenously with 1×10^{6} red blood cells infected with the CQ-resistant *P. berghei* ANKA strain were used for this assay. The infected animals were divided randomly into 10 groups of 5 animals each and were treated once daily by the oral route for 4 days with 200 mg/kg body weight of each fraction. Two controls groups were used: one treated with chloroquine 10 mg/kg body weight given daily, while the second group of animals received corn oil (vehicle solution). Parasitaemia 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.

Group I: Negative Control (received corn oil)
Group II: Positive Control (infected mice that received chloroquine 10 mg/kg body weight)
Group III-X: infected mice that received 200 mg/kg body weight of the column-fractions1-8 of the hexane extract of ADS

The percent suppression (% suppression) of parasite growth was calculated using the formula by Li et al., 2003:

$$\%\text{Suppression} = 100 - \frac{\text{Mean parasitaemia of treated animal}}{\text{Mean parasitaemia of negative control}} \times 100$$

Quantitative Beta-Hematin assay on the hexane seed extract of Aframomum danielli and its antimalarial column fractions

A colorimetric assay to determine the probable mechanism of action of the hexane extract of *Aframomum danielli* seeds and its fractions with *in vivo* antimalarial activity was performed using the beta hematin assay as described by Vargas.[9] Briefly, stock solutions of extracts and fractions were dissolved in 0.1M HCL/ MeOH / DMSO (5:3:2). The quantitative determination of the β-hematin synthesis inhibition of the extracts and fractions was done at 1.39 and 0.69 mg/mL. Ten microliter (10 µL) of the seed extract or fraction was dispensed into wells of a 24-well microtiter plate,

thereafter, 10 µL of 1M HCL was added. One hundred microliter (100 µL) of freshly prepared hematin solution was added into the wells in row A and B of the microtiter plate. The plate was shaken at 450rpm for 10 minutes. Sixty microliter (60 µL) of saturated pre-warmed acetate solution was then added to all the wells and incubated at 60ºC for 90 minutes.

Seven hundred and fifty microliter (750 µL) of 15% pyridine was then added to the first and third rows (rows A and C) while 750 µL of HEPES (Sigma) was added to the second and fourth wells (rows B and D) of the microtiter plate. The plate was shaken for 10 minutes at 450rpm and then allowed to settle for 15minutes. One hundred microliter (100 µL) from the test plate was transferred in duplicates into a 96- well plate. The absorbance was read at 405nm on a Spectramax Gemini XS microplate reader (Paul Bucher Analytik und Biotechnologie Schutzen graben 74051 Basel). The residual absorbance for each sample (ΔAAnalysis) designated (X) due to inhibition of the β-hematin was calculated using the formula: -

X = (Absorbance value in row A) - (Absorbance value in row B)

The residual absorbance (ΔACLT;Blank) designated (Y) of the sample independent from the inhibition of the β-hematin complex was calculated using the formula

Y = (Absorbance value in row C) - (Absorbance value in row D)

The resulting inhibition of the β-hematin synthesis induced by the analyzed sample was calculated using the formula: - IAnalysis = ΔAAnalysis (X) – ΔACLT:Blank (Y).

$I_{Analysis}$ values ≥ 0 are indicative of the ability of the sample to inhibit the conversion of heme to heamozoin.

Gas chromatograph/mass spectrometry of the hexane extract and fractions of Aframomum danielli seeds

The constituents of the hexane extract of *Aframomum danielli* seeds and its active column-fractions (ADHF1 and ADHF7) were determined using a gas chromatographic system (Agilent Technologies, Model 7890) fused to a silica capillary column (30m x 0.32mm) (column model HP5 MS) and equipped with a mass spectrophotometric detector (Agilent technologies, model 5975). The mobile phase was helium (99.99% purity) and was set at a flow rate of 1.2 mL /min. One microliter of the sample was injected into the column. The oven temperature was held at 80º C for one-minute holding time and raised from 80ºC to 240ºC at the rate of 10ºC/min and then held at 240ºC for 6 minutes, while the detector temperature was 250º C.

Compounds were identified by comparing the retention indices of peaks on HP5 MS column with literature values. Interpretation on the mass spectrum of individual compound was compared with the known compounds stored in the software database library, while the relative percentage of each compound was calculated by comparing the average peak area obtained for each compound to the total areas obtained.

Statistical analysis

Graph Pad prism version 5.0 (Graph-Pad Software, San Diego, CA, USA) was used for all statistical analyses. 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, $P \leq 0.05$ was considered significant.

RESULTS

In vivo antimalarial activity of the column-fractions of the hexane extract of Aframomum danielli seeds

The antimalarial activity of the column-fractions from the hexane extract, the most active of the three extracts evaluated is presented in table 1. Parasitaemia on day 4 ranged from 8.1% to 16.7% in animals treated with 200 mg/kg of the fractions while it was 0.0% and 22.4% in the treated and untreated control animals. Two column-fractions, ADHF1 and ADHF7 of hexane extract of ADS produced a significant chemosuppression of 63.9% and 62.5% respectively compared to the untreated control ($P \leq 0.05$), while fractions ADHF2, ADHF3, ADHF4, ADHF5, ADHF6 and ADHF9 produced chemosuppression ranging between 27.6 and 48.0%. The survival time of animals treated with the fractions ranged from 8 -15 days, while animals in the untreated control group survived till day 8. There was a statistically significant difference in the survival time of the animals treated with ADHF1 compared to those in the untreated group ($P \leq 0.05$). In all cases, remarkable suppression of parasitaemia by fractions translated into a longer mouse survival.

Quantitative β-hematin assay on the hexane seed extract of Aframomum danielli and its active antimalarial extracts column-fractions

The $I_{Analysis}$ value obtained for chloroquine a standard drug, the hexane extract of ADS and its active fractions was > 0 (Table 2), indicative of the ability to inhibit the conversion of heme to heamozoin. The $I_{Analysis}$ value obtained for chloroquine, a standard drug used as a control was 0.22±0.03 at 1.39 µg/mL while the $I_{Analysis}$ values obtained for the extracts and fractions ranged between 0.14±0.02 µg/mL and 0.20±0.03 µg/mL, with the hexane extract of ADS producing the highest $I_{Analysis}$ value of 0.20±0.03, which was comparable to chloroquine (Table 2). At 0.69µg/mL, chloroquine produced an $I_{Analysis}$ value of 0.51±0.02 µg/mL, while the extracts and fractions evaluated produced $I_{Analysis}$ values ranging between 0.10±0.00 µg/mL and 0.13±0.02 µg/mL (Table 2). $I_{Analysis}$ values > 0 are indicative of the ability of the extract or fraction to inhibit the conversion of toxic heme to heamozoin.

Gas chromatography of the active fractions of the hexane extract of Aframomum danielli seeds

Gas chromatography-mass spectrometry revealed the presence of monoterpenoids, diterpenoids, terpenoids, benzophenones, phenols, oxygenated sesquiterpenes and arenes in the fractions ADHF1 and ADHF7 (fractions of the hexane extract of ADS with good *in vivo* antimalarial activity) (Tables 3 and 4). The percentage of caryophyllene oxide, a terpenoid, 1H-Indene, an arene, Bis(2-ethylhexyl) phthalate, a phthalate, hexadec-2-enal, a medium chain aldehyde, 1,3,3-trimethyl-2-oxabicyclododecane, a monoterpernoid and longifolenaldeyde, a sesquiterpene in ADHF1 were 14.01%, 10.83%, 8.045%, 8.715%, 6.49% and 1.37% respectively (Table 3). The percentage of villosin, a diterpenoid, caryophyllene oxide, a terpenoid, and 3, 4, dihydroisoquinoline, 6, 7, 8-trimethoxy-1-methyl, a heterocyclic compound in ADHF7 were 11.22%, 4.71% and 7.78% respectively (Table 4).

Table 1: *In vivo* antimalarial activity of the column-fractions of the hexane extract of *Aframomum danielli* seeds against Chloroquine-resistant *P. berghei* in Swiss albino mice.

Fraction 200mg/kg	Parasitaemia ± SEM (%)	Chemosuppression ± SEM (%)	Survival time (days)
ADHF1	8.07±3.30[a]	63.93±1.22[a]	15.20±2.00[a]
ADHF2	16.76±2.77	27.58±2.74	9.40±1.03
ADHF3	14.58±1.78	37.16±1.48	9.50±1.15
ADHF4	12.92±1.91	42.32±1.04	10.20±1.40
ADHF5	13.16±1.58	41.25±0.77	10.04±0.80
ADHF6	11.64±2.17	48.04±3.58	12.60±1.80
ADHF7	8.41±2.79[a]	62.45±1.02[a]	14.80±1.40[a]
ADHF9	13.72±2.55	38.75±3.27	9.64±1.62
Corn oil	22.40±3.27	0.00±0.00	8.80±1.40
C/Q 10mg/kg	0.00±0.00	100.00±0.00	24.00±1.50

CQ = Chloroquine diphosphate (10 mg/kg/day), Corn oil (Untreated control), a = statistically significant compared to untreated control (corn oil group)

ADHF1= *Aframomum danielli* hexane fraction 1.

ADF2, 3,4, 5 e.t.c = *Aframomum danielli* hexane fraction 2, 3,4,5 e.t.c

Table 2: Quantitative evaluation of the beta-hematin inhibition activity of the hexane extract of *Aframomum danielli* seeds and its active fractions.

Extracts/fractions	$I_{\text{Analysis value}}$ 1.39 mg/mL	$I_{\text{Analysis value}}$ 0.69 mg/mL
AD hexane extract	0.20 ± 0.03	0.10 ± 0.00
ADHF1	0.14 ± 0.04	0.13 ± 0.02
ADHF7	0.14 ± 0.03	0.12 ± 0.02
Chloroquine	0.22±0.03	0.51 ± 0.02

Table 3: Phytochemical constituents of ADHF1, a column-fraction of the hexane extract of *Aframomum danielli* seeds by GC/MS analysis.

S/N	Retention Time (Minutes)	Compound	Percent of total	Class of compound/ Medicinal use
1.	7.97	Terpineol acetate	1.34	Esters/ flavour and fragrance agents
2.	8.71	4-Carene	1.03	Cycloalkanes/CNS depressant.
3.	8.86	Hexadec-2-enal	8.71	Medium-chain Aldehydes
4.	10.07	Benzene,1,2-diethyl-	3.14	Monocyclic aromatic hydrocarbon
5.	10.28	1H-1,3a-Ethanopentalen-4-ol	2.94	Monoterpene
6.	10.51	1,3 8-p-Menthatriene	1.94	Monoterpene
7.	11.37	1,4-Naplthalenediol-	1.30	Naphthalene/ antiviral, anti-inflammatory & antiplasmodial
8.	11.60	2-Caren-4-ol	1.77	Monoterpene
9.	11.86	Caryophyllene oxide	1.34	Terpenoid/antifungal
10.	12.08	Longifolenaldeyhde	1.37	Sesquiterepene
11.	12.22	8-Acetoxycarvolacetone	5.29	Terpene
12.	12.42	Caryophyllene oxide	14.01	Terpenoid/antifungal
13.	12.75	Tetra methyl-12-oxabicyclo dodeca-3,7-diene	2.16	Sesquiterpene
14.	13.42	1,3,3-trimethyl-2-	6.49	Monoterpenoids/ cough suppressant

		Oxabicyclo[2.2.2] octan-6-ol		
15.	13.88	Spiro[5.6]dodecane	1.68	Monoterpenoid
16.	13.96	2,6 –Dimethyl-3,5,7-octatriene	1.93	Terpenoid
17.	14.50	Benzoxirene-	3.26	Arene/ aromatic hydrocarbon
18.	14.67	Benzofuranone-trimethyl	0.61	Arene
19.	16.99	Hexadecanoic acid, methyl ester	1.36	Carboxylic acid/Ester
20.	17.15	3,4-hydrocouramin,	5.17	Ketones
21.	17.90	1H-Indene	10.85	Arenes
22.	18.06	5H-3-,5A,Epoxynaphth[2,1-c] oxepin	1.19	Arenes
23.	19.12	9-Octadecenoic acid,methyl ester	1.44	Carboxylic acid
24.	19.89	9-Octadecenoic acid	1.48	Carboxylic acid
25.	20.55	Pegnan-20-one,3-hydroxy-,	1.27	Sedative anaesthetic & anticonvulsant
26.	24.54	Bis(2-ethylhexyl)Phltalate	8.65	Phthalates

Table 4: Phytochemical constituents of ADHF7, a fraction of the hexane extract of *Aframomum danielli* seeds by GC/MS analysis.

S/N	Retention time (Minutes)	Compound	Percent of total	Class of compound/Medicinal use
1.	8.76	1,2,Cyclohexanediol	4.99	Alcohol substituted cyclic alcohol
2.	8.69	1,2 cyclohexandiol	14.46	Alcohol substituted cyclic alcohol
3.	12.33	Caryophyllene oxide	4.71	Sesquiterpenes/ Antifungal agent
4.	12.56	Neoisolongifolene	1.67	Halogenated terpenoids
5.	15.49	Ambrial	1.20	Aldehyde
6.	16.48	Naphthalene	2.66	Polynuclear Aromatic Hydrocarbon
7.	16.76	13- Oxabicyclo[9.3.1]	3.75	Halogenated Oxacycloalkane
8.	16.99	Hexadecanoic acid	4.44	Methyl Ester
9.	17.13	3-methoxybenzylalcohol	5.02	Anisyl alcohol
10.	17.86	5,8 di methyl-3-methylene-octahydroazulenoone	6.36	Methyl Ester
11.	19.13	9-octadecenoic acid	4.30	Methyl Ester
12.	20.73	1-Naphthalenemethanol	2.10	Bicyclic alcohol
13.	21.98	Bisoxireno[2,3:8,8a]azuleno[4, 5-b]furan-7-(3αH)-one	3.09	Sesquiterpene lactone
14.	24.17	M-(p-Chlorophenoxy)benzaldehyde	2.25	Anticancer agent
15.	24.81	Villosin	11.22	Diterpenoid

DISCUSSION

More effective, safer and relatively cheaper alternatives to the artemisinins (the bedrock of modern antimalarial chemotherapy) are urgently needed as a result of the emergence and spread of parasites resistant to the artemisinins. Ethno-Medicinal plants have proven to be a good source of antimalarial leads and drugs.[11,12] The exploration of medicinal plants that could serve as templates for the development of these alternative antimalarial agents is the foci of this study. Efforts in study were targeted at evaluating the antimalarial potential of the column-fractions of the hexane extract of *Aframomum danielli* seeds, their chemical constituents and probable mechanism of action.

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.[13,14,15] Thus, *Aframomum spp* serves as a valuable product to be explored for antimalarial activity. A previous study by Ujomu et al, showed that the hexane extract of *Aframomum danielli* seeds (ADS) demonstrated antimalarial chemosuppressive activity *in vivo*.[7]

The search for newer, safer and more effective antimalarial drugs/templates which will serve as alternatives to the artemisinins and the recent report on the potential *in vivo* antimalarial activity of the hexane extract *Aframomum danielli* seeds has necessitated the evaluation of the antimalarial activity of its column-fractions, their probable mechanism of action and their chemical constituents. Results from a previous study indicate that the hexane extracts of ADS possess good *in vitro and in vivo* antimalarial activity and were non cytotoxic to Vero cells. The ability of the hexane extract of ADS to destroy *P.berghei* parasites in Swiss albino mice and cause no harm to the Vero cells, which are normal epithelial cells of the African green monkey is an

its indicator of selective toxicity, and as such can be considered as potential lead for clinical management of malaria.

In this present study, two column-fractions (ADHF1 & ADHF7) of the hexane extract of ADS were active against the asexual erythrocytic stages of *P.berghei*, suppressing the growth of parasites at a dose of 200mg/kg body weight. A crucial prerequisite for antimalarial agents is their ability to eliminate asexual blood stage parasites.[16] The elimination of asexual blood stage parasites is a process that involves the degradation and detoxification of host heamoglobin. After the establishment of asexual blood stage parasites within a parasitophorus vacuole, heamoglobin is imported into the acidic digestive vacuole of the asexual blood stage parasite for the eventual synthesis of specific parasite proteins essential for the continued survival of the parasite.[16] The resultant effect of the digestion of heamoglobin by parasite proteases is the generation of alpha and beta chains are which are cleaved into peptides and then into amino acids.

These amino acids are essential for the generation of parasite proteins.[17] This process causes the release of Fe2+iron-containing reactive heam moieties that are oxidized inside the digestive vacuole to toxic Fe^{3+} Iron containing heam triggering oxidative stress. Within the vacuoles, Fe^{3+}iron containing heam is detoxified into heamozoin.[16] The cleavage of the endoperoxide bridge with the toxic heme which is a by-product of heamoglobin degradation, leading to a production of oxidative stress and cellular damage is a parasite specific biological process targeted by antimalarials such as the artemisinins.[16,17]
Chloroquine and amodiaquine(4-aminoquinolines) are thought to act by inhibiting the detoxification of toxic heam into heamozoin. Other parasite specific biological processes targeted by antimalarial drugs include the inhibition of folic acid synthesis by the antifolates like sulphadoxine/pyrimethamine and the inhibition of mitochondrial electron transport chain by naphthoquinones like atovaquone.[19] The mechanism of action of the hexane extract of ADS and the corresponding active column-fractions appears to be analogous to that of the aminoquinolines. The findings from the beta-hematin inhibition assay in this study shows that the hexane extract of ADS and two active column- fractions (ADHF1 & ADHF7) probably bring about their activity via the inhibition of the conversion of heam to heamozoin.

The inhibition brought about by the hexane extracts ADS and its active column- fractions probably results in a build-up of toxic heme within the food vacuole of the parasite and a production of oxidative stress leading to the death of the parasites. This build-up of toxic heme produced by the hexane extract of ADS and its active column-fractions may partly explain the observed *in vivo* antimalarial activity. From the results of this study, the hexane extract of *Aframomum danielli* seeds and its active antimalarial fractions (ADHF1 and ADHF7) may be exerting their antimalarial activity by inhibiting the conversion of a toxic by-product of haemoglobin (heam) into a non-toxic product called heamozoin necessary for the survival of the parasite.

Efforts in this study were also devoted to identifying the constituents of the active column-fractions from the hexane extract of ADS and its active column fractions using Gas chromatography/mass spectrometry. The active column fractions ADHF1 & ADHF7 contained mainly arenes and terpenoids (monoterpenes, benzophenones, oxygenated sesquiterpenes). Arenes are a group of compounds that possess antimalarial properties.[18] These compounds act by interacting with free heme, inhibiting heamozoin formation, thus preventing the growth of *Plasmodium falciparum in vitro* in a concentration-dependent manner.[18] Arenes dependently promote oxidative stress in *Plasmodium falciparum* by the generation of intraparasitic oxidants, protein carbonyls, and lipid peroxidation products. Furthermore, they deplete intraparasite GSH levels, which is essential for antioxidant defense and survival during intraerythrocytic stages.[18]

Various studies have reported the presence of terpenoids in plants with antimalarial activity.[16,17] Similarly, Aframomum species such as *A.escapum, A.latifolium, A. sceptrum and A.arundinaceum* appear to be rich in terpenoids.[13,14,15] and this is consistent with reports from our study. One of the major components of ADHF1 is caryophyllene oxide, a known oxygenated terpenoid. De Souza and colleagues reported the antimalarial activity of oleoresin which is rich in β- caryophyllene[20], thus the caryophyllene oxide present in ADHF1 may be partly responsible for the observed antimalarial activity. Naphthalenediol, a naphthalene possessing antimalarial, antibacterial, antifungal, antiviral, insecticidal, anti-inflammatory and antipyretic properties[21] is also present in ADHF1.

The analysis of *Artemisia annua* growing wild in Bulgaria shows that its essential oil contains 24.7% of caryophyllene oxide[22], while ADHF1 contains 14.01% caryophyllene oxide. Benzofuranone, an arene with good antimalarial, anti-inflammatory and anti-hypertensive properties is another compound present in ADHF1. Hexadecanoic and octadecenoic acid are two methyl esters with antimalarial activity present in ADHF1 present in small quantities. Previous studies have shown that these methyl esters which are also present in the ethyl acetate fraction of *Alchomea laxiflora*, possess antimalarial activity.[23] The presence of caryophyllene oxide, naphthalenediol and benzofuranone, compounds with known antimalarial properties within ADHF1 a fraction of the hexane extract of *Aframomum danielli* seeds might explain the antimalarial activity displayed by this fraction.

The observed *in vivo* antimalarial activity of ADHF1 might be as a result of the synergistic activity of compounds like caryophyllene oxide, naphthalenediol, benzofuranone, hexadecanoic and octadecenoic acid present in ADHF1. The fraction ADHF7, a fraction of the hexane extract of ADS, which also exhibited *in vivo* antimalarial activity contains caryophyllene oxide and arenes. The production of oxidative stress by the arenes present within the active fractions of the hexane extract of ADS may also account partly for its antimalarial activity. The production of oxidative stress by the arenes present within the active column-fractions combined with the inhibition of the conversion of toxic heme to non-toxic haemozoin might be the probable mechanism of action of the active fractions of the hexane extract of *Aframomum danielli* seeds.

CONCLUSION

The observed antimalarial activity of the hexane extract of ADS and two of its column-fractions justify its use in folklore for the treatment of febrile illnesses, thus further studies on the column-fractions in order to develop a potential antimalarial drug is necessary.

Conflict of interest

The authors declared no conflict of interest

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