

Stage -Specific Antimalarial Activity of Alkaloidal Fractions of the *Avicennia africana* P. Beauv. (Avicenniaceae) Leaf Extract

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Abstract

Background

The global call for malaria eradication rested on finding drugs that not only act against asexual but also sexual forms of the parasite. The drawbacks in disease control and prevention due to drug-resistant clones of the parasite piqued our interest in exploring for alternative antimalarial drugs from the mangrove resources.

Aims

This study evaluates the stage-specific anti-malarial and cytotoxic activities of the fractions of crude alkaloidal extracts from *Avicennia africana* leaves.

Methods

The crude and alkaloidal extracts (AAA and AAQ) from *A. africana* were fractionated using column chromatography and further analysed using GC-mass spectroscopy. The fractions were then tested for antimalarial activity against the trophozoites, schizonts, and gametocyte stages of chloroquine-sensitive strains of 3D7 *P. falciparum* using the SYBR Green 1 assay. The cytotoxic effects of the fractions were evaluated using the MTT-based assay.

Results

The fractions AAA1-AAA5 and AAQ1-AAQ5 produced promising trophozoitocidal activities with an IC_{50} value range of 0.399–45.690 $\mu\text{g/mL}$, with the artesunate (ref drug) yielding 0.09×10^{-3} $\mu\text{g/mL}$. The schizonticidal and gametocytocidal activities of selected fractions demonstrated high potency with IC_{50s} of 0.622–18.820 $\mu\text{g/mL}$ against artesunate (ref drugs) with 1.800×10^{-3} and 5.100×10^{-3} $\mu\text{g/mL}$, respectively. The cytotoxic effect of fractions produced CC_{50} that was higher than 100 $\mu\text{g/mL}$ with negligible cytotoxicity on erythrocytes and SI that ranged from 2.189 to 280.899. The major compounds identified in fractions AAA1, AAQ1, and AAQ2 were 8-carbomoylquinoline, razoxane, and dexrazoxane, respectively.

Conclusion

The fractions exhibited promising trophozoitocidal, schizonticidal, and gametocytocidal effects with no significant cytotoxic effects on RBCs. Quinoline-based alkaloids and iron chelators in this plant are implicated as possible lead-compound transmission blockers of the parasite.

Introduction

Plasmodium falciparum parasites have been reported to cause the most severe malaria, with the sub-Saharan Africa having the highest morbidity and mortality (Siagian, 2022). Malaria poses major health risk, huge economic and social burden on endemic countries (Duut & Alhassan, 2022). Even though pregnant women and children under the age of five are more vulnerable to malaria, the disease poses a greater risk to everybody (Duut & Alhassan, 2022 and Alhassan, 2021). The United Nations' Agenda for Sustainable Development Goal 3, which included total malaria eradication by 2030, may not be met due to drug resistance (DESA, 2022). This drawback necessitates a dedicated effort in the quest for alternative antimalaria drugs and other innovative techniques for the control and prevention of the infection. The *P. falciparum* exhibits an extremely complex life cycle that involves the insect vector (female *Anopheles* mosquitoes) and vertebrate host (humans) (Mianda, et al. 2022). Antimalarial drugs, particularly those that kill asexual parasites, such as artemisinin-based combination therapies, may induce or increase the production of gametocytes as a survival strategy for the parasite [Ashley et al., 2014 and Witmer et al., 2020]. Other molecular mechanisms may also stimulate the production of these gametes, leading to drug-resistant parasites, which aid in the transmission of the disease (Delves et al., 2012 and Tuteja, 2007).

Antimalarial drugs have been a pillar in the fight against malaria for years, either as chemoprophylactics or as therapeutic regimens (Imwong et al., 2017), among other control and eradication measures. However, efforts to eradicate malaria are in jeopardy due to the emergence and spread of drug-resistant clones of malarial parasites, particularly those resistant to 4-aminoquinolines such as chloroquine (CQ), sulfadoxine-pyrimethamine, and recently, artemisinin (Talisuna, et al., 2004 and WHO, 2015). Malaria control and eradication efforts are further threatened due to the invasion of *Anopheles stephensi* in urban environments in several endemic countries (Ahn, et al., 2023 and Ryan et al., 2023). Therefore, there is a need to step up preventive measures while searching for an alternative medication from natural products.

Plant medicine has been used for centuries for the treatment of various diseases, including malaria (Meshnick & Dobson, 2001 and Li & Weng, 2017). In recent years, several studies have been conducted on these plant extracts for their potential use in malarial control and elimination strategies (Raghavendra et al., 2011 and Yerbanga et al., 2014). It is a truism that many of the existing highly effective antimalarial drugs originate from natural products (Yuan et al., 2016). For example, quinine, which was used for centuries to treat malaria, was isolated from the bark of the cinchona tree. Artemisinin, another highly effective antimalarial drug, was discovered in the 1970s in a traditional Chinese herbal medicine (Dias et al., 2012, Sawyer et al., 2023 and Sinha et al., 2023). The discovery and development of safe and efficacious antimalarial medications, especially transmission-blocking ones, are essential to combating drug resistance and malaria transmission (Wadi, 2018). Several plant extracts such as *Artemisinin annua* (Chang, 2016), *Cryptolepis sanguinolenta* (Amoah, 2015), *Azadirachta indica* (Dhar et al., 1998), *Cinchona* spp. (Boratyński et al., 2019), *Carica papaya* (Mintah et al., 2022), and *Garcinia kola* (Tona et

al., 2004) were found to possess antimalarial activity against both the sexual and asexual stages of malaria parasites.

Avecinnia africana, also known as black mangrove, has been shown to have various significant ethnomedicinal properties (Thatoi et al., 2016 and Ahmed et al., 2022a). In our earlier study, we found that the ethanolic crude leaf extract of *A. africana* (AAE) had good antimalarial effects (Ahmed et al., 2022b). Crude alkaloids (AAA) and quaternary alkaloidal extracts (AAQ) from total crude (AAE) were also found to have promising antimalarial activity with good selectivity indices (Ahmed et al., 2024). However, the antimalarial activity of the plant against the different stages of malaria parasites has not been reported. To contribute to the World Health Organisation's malaria elimination programme, it is important to advance the development of antimalarial drugs with broader curative prospects that target both asexual blood and gametocyte stages (Baragaña et al. 2015). This study sought to test for trophozoitocidal, schizonticidal, and gametocytocidal effects of fractions of alkaloidal extracts from *A. africana* leaves against strains of chloroquine-sensitive 3D7 *P. falciparum* parasites using the SYBR Green 1 fluorescent assay. We further assessed the cytotoxic effects of the fractions on erythrocytes.

Material and Methods

2.1 Solvents and chromatographic materials

The following analytical grade solvents: dichloromethane (DCM), methanol (MeOH), ethyl acetate, ethanol (EtOAc), hexane, chloroform (CHCl₃) (Honeywell, Seelze, Germany), toluene (DAEJUNG chemical, China), and glacial acetic acid (Sigma-Aldrich, Steinheim, Germany) were used. Sintered-glass column (300 mm x 20mm), A TLC plate [60 F₂₅₄ (0.25mm Thick), Darmstadt, Germany], were used for the analytical thin layer chromatography. Silica gel [Aluminium oxide active neutral gel (70–230 mesh) AURO Hyderabad, India] was the stationary phase material used for the column chromatographic fractionations.

2.1.1 Plant identification and Extraction

The *A. africana* leaves were obtained from a mangrove community called Iture, near the University of Cape Coast (latitude: N5° 55.122; Longitudes: W1° 19 22.277), Cape Coast, Ghana. The specimen was identified, authenticated, and assigned a voucher number (CC3096) at the HDES, School of Biological Sciences, University of Cape Coast. Pulverized, shade-dried leaves were extracted with 70% ethanol (v/v) through cold maceration, and the filtrate was concentrated with an R-114 SABITA- rotatory evaporator at 45 rpm and 40°C to obtain total crude (AAE). The crude (AAA) and quaternary alkaloids (AAQ) were subsequently extracted from the total crude with DCM and chloroform-isopropanol mixture (3:1 v/v), respectively (Ahmed et al., 2024).

2.1.2 Fractionating alkaloidal extracts with column chromatography

The alkaloidal extracts were subjected to an aluminium oxide gel (70–230 mesh) column chromatographic technique with slight modifications (Bhimba, et al., 2010). Both crude (AAA) and quaternary (AAQ) alkaloidal extracts (3g each) were separately solubilized in a minimum amount of chloroform and mixed thoroughly with activated silica gel (5g) to ensure homogeneity and consistency. In each case, the sample was loaded on the silica gel column packed with chloroform and eluted with hexane and chloroform mixture (1:1 v/v), followed by 100% chloroform, chloroform and methanol (19:1 v/v; 9:1 v/v; 1:1 v/v; up to 1:2 v/v), and 100% methanol to yield 105 fractions of 20 mL each for AAQ and 98 fractions for AAA. All fractions were subjected to thin-layer chromatographic analysis with a mobile phase of 100% chloroform, hexane-chloroform (1:1), toluene-ethyl acetate (1:1), and chloroform-methanol (9:1). The TLC plates were visualised with a UV lamp (254 nm and 360 nm) and recorded. The eluents were combined based on their TLC profiles (retention factor (R_f)) to give five bulk quaternary alkaloidal fractions: AAQ1, AAQ2, AAQ3, AAQ4, and AAQ5. Similarly, the crude alkaloidal fractions were also bulked: AAA1, AAA2, AAA3, AAA4, and AAA5. The bulk fractions were subsequently subjected to TLC analysis on aluminium oxide gel 60 F254 (0.25mm thickness) using a developing solvent of toluene-ethyl acetate (1:1). Tables 1 and 2 show the solvent systems, R_f values, physical properties, and yields of both crude and quaternary alkaloidal fractions.

$$R_f \text{ value} = \frac{\text{Distance travelled by band}}{\text{Distance travelled by solvent}}$$

2.1.3 Gas chromatography-mass spectrometry (GC-MS) analysis of alkaloidal fractions

The profiles of AAA1, AAQ1, and AAQ2 fractions derived from crude and quaternary alkaloid extracts of *A. africana* leaves were determined using an Agilent 7890 B GC with an Agilent Technologies GC sampler 80 (Agilent Technologies, CA, USA), as previously reported (Ahmed et al., 2024). The phytochemicals detected were identified by matching the numerous peaks obtained with the NIST 2014 mass spectral library (National Institute of Standards and Technology, Mass Spectral Library).

2.1.4 Cytotoxic effects of fractions on RBCs using the MTT assay

All ten fractions were screened for cytotoxic activity against red blood cells (RBCs) using a modified version of the 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide-MTT assay (Ayisi et al., 2011). An aliquot of 100 μ L of fractions with a concentration range of 6.25 to 100 μ g/mL (two-fold serial dilution) was dispensed (in duplicate) into separate wells of a 96-well microtiter plate. In addition, 100 μ L of erythrocytes were added to each well and incubated for 3 days at 37°C in a humidified incubator (5% CO₂ and O₂). An amount of 20 μ L of 7.5 mg/mL MTT in PBS was placed in each well and kept there for 2 hours. A volume of 150 μ L of the culture media was discarded from each well. The plates were treated with 200 μ L of Triton X-100 in acidified isopropanol to dissolve any formazan formed. The plates were then kept in the dark at room temperature for 24 hours before being read at 570 nm with a plate reader.

Artesunate (Art) was used as a reference drug in this study. The concentrations at which 50% of the red blood cells were killed by the extracts (CC_{50} values) were determined by using Microsoft Excel 2016 to make a graph of the concentrations of the extracts versus the percentage of mean cell viability with dose-response curves.

The CC_{50} values were compared to standard values to find out if the fractions were harmful to cells. Also, the selectivity indices (SI) of the fractions were determined by dividing the toxic concentrations of the fractions (CC_{50}) by the effective bioactive dosages (IC_{50}). This determines the amounts of solvent fractions that inhibit or kill the parasites without causing deleterious consequences to the host cells (RBCs).

2.2 Biological drug Assay

2.2.1 Cultivation of ring-stage parasites and adaptation

The antimalarial effects of solvent fractions of alkaloidal extracts (AAA and AAQ) were evaluated on the chloroquine-sensitive clone of 3D7 *P. falciparum*. The asexual parasites were maintained in cultures *in vitro* using the method described by Trager & Jensen [Trager & Jensen, 1976]. with slight modifications. The parasites were cultivated in human erythrocytes (O + RBC, 4% haematocrit) in a complete parasite medium (CPM), which comprises 10.44 g/L RPMI 1640, 5.94 g/L HEPES, 5 g/L AlbuMAX II, 50 mg/L hypoxanthine, and 2.1 g/L sodium bicarbonate. The T75 culture flasks were incubated at 37°C in an atmosphere of 93% N₂, 4% CO₂, and 3% O₂ amidst daily medium changes to obtain more than 5% parasitaemia. Light microscopy (100x oil immersion) of 10% Geimsa-stained slides (thin films) was done every day to estimate the percentage of parasitaemia (Fig. 1A), which was used to track parasite growth until more than 5% parasitaemia was recorded (Lambros & Vanderberg, 1979). To do the plating, 10 mL of a parasite suspension (2% haemoglobin, 1% parasitaemia) that had been made earlier in CPM with noninfected red blood cells was used.

2.2.2 Screening of fractions for antimalarial activities at the trophozoite stage

All the solvent fractions of the alkaloidal extracts were screened for antimalarial activity using the SYBR Green 1 assay. In this study, all ten fractions (3 mg each) were dissolved separately in 3 mL of 0.5% DMSO (v/v) to yield a stock concentration of 1000 µg/mL. The stocks were again serially diluted (10-fold) to obtain a working concentration of 100 µg/mL in CPM. Furthermore, the working solution was further diluted (2-fold) to obtain concentrations ranging from 100 to 0.39 µg/mL. To assess the inhibitory effects of the fractions, a 96-well costar plate was filled with 100 µL of each dilution in duplicates, alongside the reference drug (artesunate, 15 ng/mL). After that, each well was treated with 100 µL of infected erythrocytes at 2% PCV with 1% parasitaemia (> 90% sorbitol-synchronized ring stage parasites) suspension from the 2nd to the 10th well. The negative control wells (the 11th well) were filled with 100 µL of parasite suspension without fractions. The same method was used for the rest of the fractions.

The plates were then put in a modular chamber, gassed for 5 minutes with a mixture of 5% O₂, 5% CO₂, and 90% N₂, and kept at 37°C for 72 h (Kumatia, et al., 2023).

An aliquot of 100 µL of buffered SYBR Green 1 dye (20 mM Tris-HCl, 5 mM EDTA, 0.008% saponin in PBS, pH 7.5 and 0.08% Triton X-100) was added to each well, mixed gently, and incubated for 30–60 minutes in the dark. The plates were read using the FLUOstar OPTIMA Fluorometer Plate Reader (control software version 2.20) at 470 nm excitation and 520 nm emission. To find the half-maximal inhibitory concentration (IC₅₀) for each fraction, we used non-linear regression analysis on dose-response curves.

2.2.3 *In Vitro* Production of Schizonts

The ring-stage parasites were kept in a continuous culture until parasitaemia was greater than 8%, with most of the parasites being schizonts. The parasites were then synchronized with 60% Percoll (30 mL Percoll + 17 mL RPMI + 3 mL 10x PBS). The parasite culture in a 15-mL falcon tube was centrifuged at 600g for 5 minutes at room temperature. The resultant pellet was washed twice in incomplete RPMI. The final pellet was suspended in a complete parasite medium to obtain 20% haematocrit. A volume of 7 mL of 60% Percoll was measured into a 15-mL falcon tube, layered slowly with 2.5 mL of the parasite suspension, and centrifuged at 700g for 10 minutes. The schizonts, which formed a distinct reddish-brown band at the top of Percoll, were transferred to a new tube. Thin smears were made, stained with 10% Geimsa stain for 20 minutes, and examined under an oil immersion (100x) objective lens to estimate parasitaemia (Fig. 1B). The culture was adjusted to 2% haematocrit with fresh, uninfected erythrocytes before 20 mL of CPM (parasite suspension) was used for plating with the selected fractions (Kumatia, et al., 2023).

2.2.4 Schizonticidal activity of selected fractions

The efficacy of the fractions was further assessed on the asexual schizont forms of the 3D7 parasites. In this study, the SYBR Green 1 assay was used to evaluate the schizonticidal activity of fractions that had shown promising antiplasmodial effects at the trophozoidal stage. Concisely, an aliquot (100 µL) of various serially diluted concentrations (100–0.39 µg/mL) of the fractions was seeded in 96-well plates in duplicates alongside the reference drug (15 ng/mL artesunate) as described above. One hundred microliters of percoll-synchronized schizont suspension (2% PCV and 1% parasitaemia) were added to each treated well (2nd well to 10th well). In addition, the 11th well of the costar plates was also seeded with 100 µL of predominantly schizont parasite suspension without the fractions to serve as a negative control. The plates were then gassed for 5 minutes with gas mixture (90% nitrogen, 5% oxygen, and 5% carbon dioxide) in a modular chamber and incubated at 37°C for 72 hours. The plates were harvested after 72 h and the SYBR Green 1 assay was performed and read using the plate reader as previously reported (Kumatia, et al., 2023).

2.2.5 *In Vitro* Culturing of Gametocytes

A volume of two hundred microliters of infected RBCs (5% PCV and 1% Parasitaemia) was aliquoted into a new T-25 cm³ flask containing 5 mL of CPM to initiate gametocyte production, as proposed earlier with

some modifications [(Kumatia, et al., 2023 and Wadi et al., 2019)]. Culture was allowed to grow amidst media changes, and the growth of parasites was checked by microscopy daily. The culture was then transferred into a new T-75 cm³ flask and kept in the incubator after parasitaemia was above 8%. At 10% parasitaemia (ring-stages), the culture was synchronized with 5% sorbitol to obtain only rings and was then treated with freshly prepared CPM and 50 mM N-Acetyl Glucosamine (NAG). This was designated as day zero.

Consequently, the flask was flushed with a gentle flow of gas, then capped tightly, and returned to the incubator. From days one to four, the spent media was removed and discarded, and CPM and 50 mM N-acetylglucosamine (source) were added to the flask, gassed, and kept in the incubator. NAG was added to the culture not only to eliminate or suppress asexual parasites but also to thwart the emergence of new gametocytes. The spent media was discarded on day five, and only CPM was added to the flask at this stage and then gassed before returning to the incubator. The procedure was repeated until day fourteen to ensure most of the parasites in the culture were mature gametocytes (1V and V). The percentage of gametocytes was estimated by counting the number of gametocytes per 1000–1500 RBCs (Figs. 1C and 1D). A gametocyte suspension (2% haematocrit and 1% parasitaemia) was prepared, which was later used to test for the inhibitory effects of the selected fractions.

2.2.6 The Gametocytocidal Activity of Fractions

Here, the SYBR Green 1 assay was used to determine the inhibitory effects of fractions on gametocytes. Briefly, the fractions that showed promising activity at the trophozoite and schizont stages were used in this study. One hundred microliters of the fractions that were 2-fold diluted (100 – 0.39 µg/mL in CPM) were pipetted into a 96-well plate in duplicate. The reference drug (15 ng/mL Artesunate) was plated alongside the most potent fractions. An aliquot of one hundred microliters of gametocyte suspension (2% PCV and 1% parasitaemia) was added to each well, along with a negative control (gametocyte suspension without fraction) with the same volume set at the 11th well. The plates were gassed for 5 minutes with the same gassing conditions as above and kept at 37°C for 72 h in a modular chamber. After the incubation period, each well was treated with 100 µL of buffered SYBR Green 1 dye, mixed carefully, and left in the dark for 30 to 60 minutes before reading the plates as described earlier (Kumatia, et al., 2023).

2.3 Grading of Plant Extracts' Antimalarial Efficacy against *P. falciparum*

The categorization of potential antimalarial drug candidates in active fractions in this study was based on IC₅₀ values; the lower the value, the more potent the plant extract is against the parasite, as proposed by Kamaraj et al. (Kamaraj et al., 2012). Plant extracts of IC₅₀ less than 10 µg/mL were classified as having a promising activity; those between 10 and 20 µg/mL were categorised as moderate; and extracts possessing 20–40 µg/mL were classified as having good activity. Extracts scored with IC₅₀ values between 40 and 70 µg/mL were considered marginally potent, and extracts with IC₅₀ values greater than 70 µg/mL were classified as poor.

2.4 Statistical analysis

The concentrations of fractions that inhibit both sexual and asexual *P. falciparum* by 50% (IC₅₀) were estimated from dose-response curves using non-linear regression analysis with GraphPad Prism version 7.0 software (GraphPad Software, San Diego, CA, USA). Using Microsoft Excel 2016, a dose-response curve was determined, and the 50% cytotoxic concentration (CC₅₀) was calculated. The results were presented as the mean ± standard deviation (SD), with statistical significance set at $p < 0.05$.

Results

3.1 Extraction yield and physical properties of fractions

Tables 1 and 2 demonstrate the fractionation findings of alkaloidal extracts (AAA and AAQ) that were previously extracted from the total crude (AAE, 70% ethanol, cold maceration) of *A. africana* leaves and reported (Ahmed et al., 2024). The various physical properties of fractions, such as colour and consistency, were not significantly different from what had previously been described regarding both the total crude and the alkaloids (Ahmed et al., 2024). Out of the ten (10) bulked fractions, AAQ1 and AAQ2 formed chalky white to colourless crystals (Fig. S1).

Table 1
Physicochemical properties of alkaloidal fractions.

Fractions	Solvents	R _f Values	Consistency	Colour	Weight (mg)	Yield (% w/w)
AAA1	Hexane/CHCl ₃	0.92	Gummy	Brown	20.203	0.673
AAA2	CHCl ₃	0.98	Semi solid	Brown	32.714	1.090
AAA3	CHCl ₃ /MeOH	0.97	Gummy	Greenish brown	8.702	0.290
AAA4	CHCl ₃ /MeOH	0.97	Gummy	Dark	9.034	0.323
AAA5	CHCl ₃ /MeOH	0.95	Gummy	Greenish brown	35.801	1.193
AAA- <i>Avicennia africana</i> alkaloidal fractions 1–5						

Table 2
Physicochemical properties of alkaloidal fractions.

Fractions	Solvents	R _f Value	Consistency	Colour	Weight (mg)	%Yield (w/w)
AAQ1	Hexane/CHCl ₃	0.16	Crystal	Chalky white to colourless	19.412	0.647
AAQ2	CHCl ₃	0.13	Crystal	colourless	23.905	0.797
AAQ3	CHCl ₃ /MeOH	NAD	Gummy	Brown	9.820	0.327
AAQ4	CHCl ₃ /MeOH	0.97	Gummy	Brown	27.303	0.910
AAQ5	CHCl ₃ /MeOH	0.97	Gummy	Golden brown	28.910	0.964

AAQ: *Avicennia africana* quaternary alkaloidal fractions (1–5); NAD: not determined.

3.1.2 GC-MS identification of compounds in fractions (AAA1, AAQ1, and AAQ2) of alkaloidal extracts of *A. africana*

The GC-MS evaluation of selected active fractions shows the presence of various phytochemicals inherent in the plant. There were seven (7) compounds identified in the fraction AAA1, with three isomers of 8-carbamoylquinoline having the highest peak area percentage of 95.43 (Figure S2 and Table S1). The GC-MS analysis of AAQ1 and AAQ2 fractions showed various active principal compounds (Tables S2 and S3, Figures S3 and S4). In the AAQ1 fraction, three phytochemicals were identified, with razoxane recording the highest peak area of 90.54 percent. In AAQ2, the most abundant compound found in the fraction showed one main phytochemical of dextrazoxane with percentage abundance peak area of 62.12% followed by two isomers of methanesulfonamide, N-[2-[ethyl(3-methylphenyl) amino]ethyl] (5.87%) (Table S3 and Fig. S4).

3.2 Trophozoitocidal and Cytotoxic effects of solvent fractions

The suppressive effects of the ten active fractions against the asexual stages of the 3D7 *P. falciparum* parasites are shown in Tables 3 and 4. Out of five solvent fractions of the alkaloidal extracts (AAA1–5, hexane/CHCl₃/MeOH), AAA1 exhibited very strong antimalarial effects with an IC₅₀ value of 0.399 µg/mL. Also, AAA3 was the second strongest with an IC₅₀ value of 0.563 µg/mL, with AAA5 registering the least IC₅₀ value of 18.140 µg/mL (Table 3). The erythrocytes showed a high survival rate when exposed to various concentrations of the fraction, like the positive control. All fractions (AAA) had good selectivity indices (5.513 < SI < 250.636) towards the parasites at both the ring stages as well as the mature trophozoite stages.

The five crude quaternary alkaloidal (AAQ1–5, hexane/CHCl₃/MeOH) fractions demonstrated similar inhibitory patterns against 3D7 *P. falciparum* trophozoites parasites, as summarised in Table 4. In the order of high antimalarial efficacy with good selectivity indices, AAQ4 (IC₅₀ = 0.356 µg/mL, SI = 280.899) ranked promising, followed by AAQ3 (0.475 µg/mL, SI = 210.526). The AAQ2 and AAQ1 fractions also showed promising trophozoitocidal activity against the parasites, with IC₅₀ and selectivity indices of (0.589 µg/mL, SI = 169.779) and 1.582 µg/mL, SI = 63.211, respectively. However, AAQ5 (45.690 µg/mL) showed marginally potent activity against 3D7 parasites with a selectivity index of 2.189.

Table 3

Trophozoitocidal activities, toxic effects on erythrocytes and selectivity indices of alkaloidal fractions (AAA1-5) of *A. africana* leaf

Fractions	Antiplasmodial efficacy against 3D7 <i>P. falciparum</i> IC ₅₀ ± SD (µg/mL)	Cytotoxicity against RBCs CC ₅₀ (µg/mL)	Selectivity indices CC ₅₀ /IC ₅₀
AAA1	0.399 ± 0.045	> 100	250.626
AAA2	14.990 ± 0.014	> 100	6.671
AAA3	0.563 ± 0.027	> 100	177.620
AAA4	2.843 ± 0.048	> 100	35.174
AAA5	18.140 ± 0.028	> 100	5.513
Artesunate	0.09 ± 0.03 (x10 ⁻³)	> 100	1111 (x 10 ³)
Values presented as mean ± standard deviation (SD), n = 2			

Table 4

Trophozoitocidal activities, toxic effects on erythrocytes and selectivity indices of alkaloidal fractions (AAQ1-5) of *A. africana* leaf

Fractions	Antiplasmodial efficacy against 3D7 <i>P. falciparum</i> IC ₅₀ ± SD (µg/mL)	Cytotoxicity against RBCs CC ₅₀ (µg/mL)	Selectivity indices CC ₅₀ / IC ₅₀
AAQ1	1.582 ± 0.036	> 100	63.211
AAQ2	0.589 ± 0.026	> 100	169.779
AAQ3	0.475 ± 0.089	> 100	210.526
AAQ4	0.356 ± 0.024	> 100	280.899
AAQ5	45.690 ± 0.386	> 100	2.189
Artesunate	0.09 ± 0.03 (x10 ⁻³)	> 100	1111 (x 10 ³)
Values presented as mean ± standard deviation (SD), n = 2			

3.2.1 Schizonticidal and gametocytocidal activities of active fractions

The antimalarial activity of the selected active fractions against schizonts and gametocyte stages of 3D7 *P. falciparum* parasites, which were tested on the trophozoite stages, showed variable potency. In the schizonticidal activity study, all the selected fractions produced high activity, with IC₅₀^{Pf} values between 0.622 and 3.322 µg/mL, in the following order of activity: AAA3 < AAQ4 < AAA4 < AAA1 < AAQ1 < AAQ2, (Table 5). The inhibitory activities of fractions on mature gametocytes demonstrated equally good gametocytocidal effects in a dose-dependent manner. The fraction AAA4 gave the highest activity (IC₅₀ value of 5.030 µg/mL), while AAQ4 exhibited a low IC₅₀ value of 18.820 µg/mL compared to Artesunate.

Table 5
Schizonticidal and gametocytocidal activities of selected active fractions of alkaloidal extracts from *A. africana* leaf

Fractions	Schizonticidal efficacy against 3D7 <i>P. falciparum</i> IC ₅₀ ± SD (µg/mL)	Gametocytocidal efficacy against 3D7 <i>P. falciparum</i> IC ₅₀ ± SD (µg/mL)
AAA1	1.712 ± 0.0059	5.219 ± 0.0059
AAA3	0.622 ± 0.0051	5.820 ± 0.0108
AAA4	0.904 ± 0.0047	5.030 ± 0.0091
AAQ1	3.265 ± 0.0019	14.100 ± 0.0299
AAQ2	3.322 ± 0.0022	8.460 ± 0.0014
AAQ4	0.797 ± 0.0046	18.820 ± 0.0258
Artesunate	1.800 ± 0.0294 (x10 ⁻³)	5.100 ± 0.0571 (x10 ⁻³)
Values presented as mean ± standard deviation (SD), n = 2		

3.3 Discussion

The search for alternative and complementary antimalarial drugs for treatment and the quest for elimination using selected plant extracts have been gaining attention in drug research. Previous studies on plant extracts have primarily focused on the stage-specific activities crucial for the elimination of malaria (Dhar et al., 1998, Boratyński, 2019; Mintah et al., 2022; Tona et al., 2004 and Kumatia et al., 2023). These studies have over time shown high antimalarial activities; the outcome of our current study was similar. In the fight against malaria, natural products with high activity against *Plasmodium* parasites cannot be overlooked since plants are endowed with enormous bioactive compounds (Kan et al., 2023). In this study, we examined the mangrove resource, emphasising the trophozoitocidal,

schizonticidal, and gametocytocidal potentials of *A. africana* plant. We earlier reported the antimalarial effects of an ethanolic crude extract of *A. africana* leaf AAE (Ahmed et al., 2022b), and subsequently extracted crude and quaternary alkaloids (AAA and AAQ) from the total crude, which had promising antimalarial and antioxidant activities (Ahmed et al., 2024).

In this investigation, we fractionated the alkaloidal extracts AAA and AAQ into fractions (AAA1- AAA5 and AAQ1-AAQ5) and screened against both asexual and sexual stages of the chloroquine-sensitive (3D7) *P. falciparum* parasite clone [40]. It is worth noting that seven (7) of the active fractions have shown potentially promising trophozoitocidal activities, with AAA5 and AAQ5 showing moderate and marginally potent trophozoitocidal effects, respectively. The IC₅₀ values of the fractions against the ring stage and the mature trophozoites, in order of decreasing potency, are: AAQ4_{0.356} < AAA1_{0.399} < AAQ3_{0.475} < AAA3_{0.563} < AAQ2_{0.589} < AAQ1_{1.582} < AAA4_{2.843} < AAA2_{14.990} < AAA5_{18.140} < AAQ5_{45.690} (Tables 3 and 4). The trophozoitocidal effects of the fractions may be due to the combined or single impacts of the biologically active properties of the numerous metabolites in these fractions, or they may be due to the single effect of an individual phytocompound in the plant.

The various phases of the parasite's life cycle are important in its transmission to mosquito vectors. As a result, interrupting this process at any point during its life cycle is critical for successful therapy and management (Yu et al., 2022). In this study, all the fractions have shown high inhibitory effect on the trophozoite stage, which plays an integral role in the disease pathology and clinical symptoms. It is important that therapeutic agents target the trophozoite stage in the parasite's life cycle, which may be critical to preventing the emergence of drug-resistant strains.

The erythrocytic schizogonic cycle in the human host is implicated in the pathology associated with malaria. The cycle starts with the invasion of erythrocytes by merozoites to undergo a trophic period, which culminates in early trophozoites becoming mature trophozoites. Multiple rounds of nuclear division, resulting in the formation of schizonts, mark the end of the trophic period (Mianda et al., 2022). It is important to note that the synchronous rupture of the infected erythrocytes (schizonts) results in the intermittent fever paroxysm in the other malaria species, with *P. falciparum* often exhibiting a continuous fever.

The parasite sustains and perpetuates transmission through its complex life process between humans and mosquitoes. In gametocyte formation, a minute set of committed asexual parasites (< 10%) differentiates into sexual forms (gametocytes), the transmissible forms that infect the mosquito vector (Mianda et al., 2022). The immature gametocytes are sequestered in the bone marrow tissue and spleen (De Niz et al., 2018 and Obaldia 3rd et al., 2018) to further undergo a maturation process from stages I to V, which is believed to last for 10 days in the vertebrate host (Hawking et al., 1971). The mature gametocytes (stage V) are then released into the peripheral blood, where they are picked up by mosquitoes during a blood meal (Venugopal, et al., 2020).

Reports have shown that gametocytes and asexual stages of *P. falciparum* parasites react differently to antimalarial drugs in malaria treatment (Delves et al., 2013). However, all the selected trophozoitocidally active fractions from the screening in this study yielded promising schizonticidal and gametocytocidal activities, except for AAQ1 (14.100 µg/mL) and AAQ4 (18.820 µg/mL) fractions that had moderate gametocytocidal activities (Table 5). An improved treatment outcome is anchored on an effective antimalarial drug with schizonticidal effects. Such a drug should drastically reduce the symptoms associated with infection and avert the sequestration of infected RBCs in the brain microvasculature, which could cause cerebral malaria. In this study, the fractions have shown promising schizonticidal effects and inhibited the parasite's gametocyte stage, which eventually causes disruptions in the parasite's life cycle. This study supports previous investigations regarding plant extracts with gametocytocidal and asexual stage activities (Amoah et al., 2015; Chang, 2016; Dhar et al., 1998; Boratyński et al., 2019 and Mintah et al., 2022).

The major phytocompound identified in AAA1 in the GC-MS spectral analysis was 8-carbomoylquinoline, with a peak area of 94.24%, along with other compounds in trace amounts. The 8-carbomoylquinoline compound identified in this fraction is synonymous with quinoline-8-carboxamide, a quinoline derivative with a benzene ring fused to a pyridine ring that exhibits a wide range of biological activities. Baragaña and collaborators had previously reported the antimalarial activity of quinoline-4-carboxamide, which kills the parasite by blocking elongation factor 2 (*PfeEF2*) in the parasite protein synthesis (Baragaña et al., 2015). Other researchers have confirmed the antimalarial activity and mechanism of action of this compound (Schneider et al., 2023 and Umumararungu et al., 2023).

The difference between quinoline-4-carboxamide (C = 4) and quinoline-8-carboxamide (C = 8) is the position of the carboxamide group on the quinoline ring. The location of functional groups on the aromatic ring can influence the compound's specific chemical properties as well as its biological activities. It is important to note that the activity of antimalarial drugs can be influenced by some factors, such as the compound's overall structure, electronic properties, and stereochemistry. Based on this, we can suggest that the trophozoitocidal, schizonticidal, and gametocytocidal effects seen in AAA1 might be due to the unique action of 8-carbomoylquinoline. There is also the possibility that the antimalarial activities may be due to the synergy of the trace compounds and the major compound in the fraction, as far as breaking the life cycle of the parasite with these active fractions is possible.

The GC-MS spectral analysis of AAQ1, and AAQ2 revealed several phytocompounds (Tables S2 and S3). Razoxane was found as the major phytocompound (90.56% peak area) identified in AAQ1 fraction together with four other compounds in trace amount having percentage peak areas that ranged from 0.94 to 2.81. On the other hand, dexrazoxane (62.12% peak area) was identified together with other phytocompounds in AAQ2. Razoxane and dexrazoxane are two very related compounds that have been largely investigated for their anti-cancer and cardioprotective properties (Diwanay et al., 2004). Interestingly, we previously identified this pair of stereoisomeric compounds from crude quaternary alkaloids (AAQ) and found that they not only exhibited promising trophozoitocidal effects but also antioxidant properties and were non-toxic against RBCs (Ahmed et al., 2024). Similarly, the present

investigation found that all the fractions had promising trophozoitocidal and schizonticidal activities. Out of the six selected fractions, four showed promising gametocytocidal potency, with two fractions demonstrating moderate gametocytocidal effects. A previous study has shown that, razoxane and dexrazoxane identified in the fractions of AAQ1 and AAQ2 were iron-chelator prodrugs that prevents the cardiopathic effect of anthracycline drugs in cancer treatment (Hasinoff et al. 2003). However, these compounds were proposed to have antimalarial effect when they are hydrolyzed to iron-chelating intermediates (Loyevsky et al., 1999). It has also been postulated that quinoline-based antimalarials kill the parasites by inhibiting or blocking the hemozoin formation at the hemocrystal growth sites in the digestive vacuole (Sullivan Jr, 2017). Interestingly, iron chelators have been suggested to disrupt hemozoin formation, resulting in the buildup of toxic haem, which causes parasite damage. In this study, dexrazoxane may have reduced iron availability, interfered with haem detoxification, and inhibited parasite growth and replication within host erythrocytes. Certain iron chelators, such as FBS0701, have been reported to display antimalarial effects at both sexual and asexual stages *in vitro* and *in vivo*, but their use was restricted owing to poor pharmacokinetic qualities (Ferrer et al., 2012). Hence, the idea of considering iron chelator prodrugs as antimalarial drugs that target iron metabolism in *Plasmodium* parasites seems promising (Pradines et al., 2002).

In a malarial control strategy, any effective and safe antimalarial agents must protect host erythrocytes from haemolysis (Ahmed et al., 2024). In this context, the CC₅₀ values for all active fractions in the cytotoxicity experiment were greater than 100 µg/mL, indicating RBC survival was significantly higher than 50% (Tables 3 and 4). This suggests that all the fractions screened using the MTT-based assay were nontoxic or had negligible cytotoxic effects on host erythrocytes. In malaria treatment, the selectivity of antimalarials targeting the parasite specifically and not the human RBCs is of high importance (Ayisi, et al., 2011). Hence, drugs with high selectivity indices (SI) are very useful to reflect a more beneficial safety profile of the drug, with low toxic effects on humans and high potency against parasites (Ayisi, et al., 2011). The active fractions in our current study yielded SI > 2 (64) (SI = 2.189-280.899), particularly for AAA1 (8-carbomoylquinoline SI = 250.626), AAQ1 (razoxane, SI = 63.211), and AAQ2 (dexrazoxane, SI = 169.779). The selectivity of the active fractions against trophozoites, schizonts, and gametocytes of the (3D7) *P. falciparum* clones as compared to non-infected RBCs was promising. The selectivity indices in this study were greater than 2, as Appiah-Oppong et al. suggested for natural products Appiah-Opong et al., 2011), demonstrating the safety of these fractions. The identification of razoxane and dexrazoxane in the fractions (AAQ1 and AAQ2) of quaternary alkaloids from *A. africana* in the present study supports and lends credence to our previous study (Ahmed et al., 2024).

The concept of iron-chelator prodrugs and quinoline-based alkaloids and their application in malaria control strategies targeting both sexual and asexual stages of parasites may be the game changer in the malarial elimination drive. We recommend that subsequent studies consider the isolation, characterization, and modification of these compounds. In addition, an antimalarial efficacy study of the fractions against other parasite cell lines may be recommended to confirm the stage-specific activity as obtained in this study. Though findings support folklore usage, outcome may not be clinically

translatable as the protocol employed in the *in vitro* antimalarial investigation may have some limitations.

Conclusion

The study underscores the potential of *A. africana* as valuable resource for the discovery of novel antimalarial agents. This research has demonstrated that fractions of crude (AAA) and quaternary (AAQ) alkaloidal extracts possess promising antimalarial activity against the trophozoites, schizonts, and gametocytes of 3D7 *P. falciparum* parasites. Importantly, the fractions demonstrated promising potency while exhibiting minimal cytotoxicity, thus suggesting a favourable therapeutic agent. In addition, the identification of 8-carbomoylquinoline, razoxane, and dexrazoxane, respectively, with potential transmission-blocking activities further provides insights into the chemical constituents responsible for the observed results. The erythrocyte viability assay has shown that the fractions have negligible cytotoxic effects against human erythrocytes, with most of them yielding high selectivity indices for the asexual and sexual stages of the parasites. Therefore, further isolation, characterization, and structural modification of these quinoline-based alkaloids and dexrazoxane compounds can lead to the creation of a new class of antimalarial drugs with ideal potency against both the sexual and asexual forms of *Plasmodium* parasites.

Abbreviations

AAA: Crude alkaloids

AAQ: Quaternary alkaloids

FBS0701: (S)3''-(HO)-desazadesferrithiocin-polyether-DADFT-PE

HDES: Herbarium of the department of environmental sciences

Declarations

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Ethical Approval

This declaration is “not applicable”.

Competing interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants patents received or pending, or royalties. The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Consent to Participate and Consent to Publish

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Authors' contributions

All authors (Mustapha A. Ahmed, Francis Ackah-Armah, George Ghartey-Kwansah, Patrick M. Fynn, Felix K. Zoiku, Ebenezer Ofori-Attah and Christian K. Adokoh) contributed to the study conception and design. Material preparation, data collection and analysis were performed by Christian Kweku Adokoh and Mustapha A. Ahmed. The first draft of the manuscript was written by Mustapha A. Ahmed and co-authors' (Francis Ackah-Armah, George Ghartey-Kwansah, Patrick M. Fynn, Felix K. Zoiku, Ebenezer Ofori-Attah and Christian K. Adokoh) commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data and materials are clearly stated in the manuscript for understanding.

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Figures

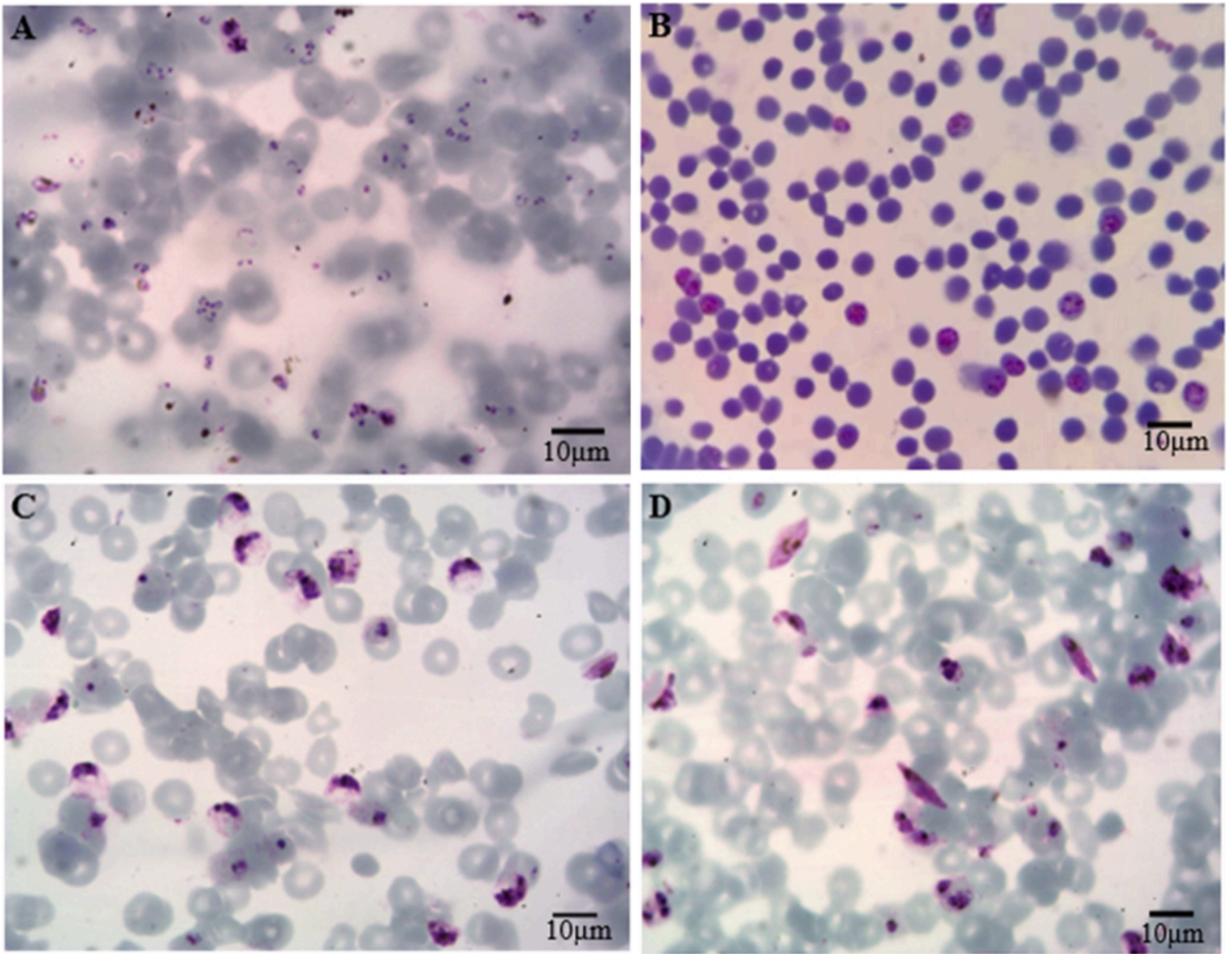


Figure 1

Developmental stages of *Plasmodium falciparum* in *in vitro* cultures: **A.** shows early and mature trophozoites, **B.** represents schizonts, and **C.** and **D.** show early and late gametocytes in continuous *in vitro* cultures (1000x, 10% Giemsa stain).

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