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Evaluation of the Larvicidal, Antiplasmodial and Cytotoxicity Properties of *Cassia arereh* Del. Stem Bark

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Authors' contributions

This work was carried out in collaboration between all authors. Author HI performed the analysis and managed the literature searches. Author AEWHAA performed the statistical analysis. Author SMY wrote the protocol, designed the study, managed the discussion and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The present study was carried on the stem bark of *Cassia arereh* Del. Different extracts were prepared and were assessed for their *in vitro* larvicidal, antiplasmodial and cytotoxicity properties.
Methodology: Larvicidal activity of the methanol extract was evaluated against 3rd instar larvae of *Culex quinquefasciatus* using method recommended by World Health Organization. The antimalarial activity of chloroform, ethyl acetate, methanol and aqueous extracts was assessed on the chloroquine-resistant strain W2 strain of *Plasmodium falciparum* by flow cytometry. Cytoxicity effect was assessed on two human cell lines K562S (by flow cytometry) and HepG2 (by MTT assay).
Results: The larvicidal activities of different concentrations of *C. arereh* stem bark methanolic extract after 24 h of incubation revealed that the 1.0 g/L concentration gave the highest mortality (40.2%). lethal concentrations that killed 25% (LC_{25}), 50% (LC_{50}), 90% (LC_{90}) and 95% (LC_{95}) of larvae in 24 h were 4235, 8230, 16007, 29325 and 40680 ppm. The best antimalarial activities were detected in the chloroform extract with IC_{50} > 12.5 µg/ mL and cytotoxicity effect with IC_{50} > 12.5 µg/ mL on both K562S and HepG2 cell lines indicative of promising security index. Phytochemical screening indicated the presence of anthraquinones, flavanoids, terpenes, steroles and tannins.

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Conclusion: This result indicates that *C. arereh* stem bark could be a good source of bioactive ingredients.

Keywords: Cassia arereh; larvicidal activity; antiplasmodial activity; cytoxicity.

1. INTRODUCTION

Mosquitoes serve as vectors of several diseases causing serious health problems to humans and development of resistance towards chemical insecticides initiated a search for alternative control measures [1]. *Anopheles* is an important vector for the transmission of malaria [2,3], *Aedes* is known for the transmission of yellow fever [4] and *Culex* is known for transmission of filariasis in human and lumbar paralysis in cattle [5,6]. Malaria and filariasis rank amongst the world most prevalent tropical infectious diseases. An estimated 300–500 million people are infected with malaria annually, resulting in 1.5–3 million deaths [7]. Malaria remains a major health problem in Sudan. Accordingly, about 20–40% of outpatient clinic visits and approximately 30% of total hospital admissions are due to malaria [8]. Lymphatic filariases (LF) is probably the fastest spreading insect-borne disease of human in the tropic, about 30% (394 million) of the global population are estimated to be in the LF endemic countries of the African region [9].

Plasmodium falciparum the most widespread etiological agent for human malaria has become increasingly resistant to standard antimalarials e.g. chloroquine and antifolates [10]. Consequently, new drugs or drug combinations are urgently needed today for the treatment of malaria. These drugs should have novel modes of action or be chemically different from the drugs in current use.

Plants offer an alternative source of insect-control agents because they contain a range of bioactive chemicals [11], many of which are selective and have little or no harmful effect on non-target organisms and the environment. Much effort has, therefore, been focused on plant extracts or phytochemicals as potential sources of mosquito control agents or as lead compounds [12,13].

In Africa and elsewhere, plant extracts are still widely used in the treatment of malaria and other ailments, and up to 80% of the African population use traditional medicines for primary health care [14]. Since little scientific data exist to validate antimalarial properties of these medicinal plants, it is important that their claimed antimalarial properties are investigated, in order to establish their efficacy and determine their potential as sources of new antimalarial drugs.

Cassia arereh Del. (Fabaceae) is a shrub or small tree widely spread in short grass Savannah throughout Central and Southern Sudan. Decoction of the stem bark is used to treat diarrhoea, stomach ache and malaria [15]. Also, in Nigeria, the root and the stem bark are used in disease conditions such as diarrhoea, dysentery, stomach ache, ascites, headache, cough, rheumatism, back pain, wound healing, weakness, avian plague, yellow fever and malaria [16]. Not much has been reported on the biological properties of this plant. Thus, the objective of this study was to investigate the larvicidal activity against *Cx. quinquefasciatus*, antiplasmodial and cytotoxic properties of *C. arereh* stem bark extracts.

2. MATERIALS AND METHODS

2.1 General

Cell culture medium (RPMI 1640), foetal calf serum, L-glutamine, non essential amino acids and other medium additives were from Eurobio (Paris, France). All other chemicals were of highest chemical purity and were purchased from Sigma except contrary mention. Stock solutions of quinazoline derivatives were prepared in DMSO. Stock solutions of reference drugs (doxorubicin, chloroquine and doxycycline) were prepared in ultrapure H_2O or DMSO. Flow cytometry was performed using a FACS sort flow cytometer apparatus (Beckton Dickinson, Paris, France), equipped with an argon laser (power of 15 mW, and wavelength of 488 nm).

2.2 Plant Material

The stem bark of *C. arereh* was collected from the Blue Nile (Eldamazeen) State in East South Sudan. The plant was authenticated at the Herbarium of Botany Department, University of Khartoum where a voucher specimen, No. NBH/CA209, was deposited. The stem bark was dried under shade, ground into soft powder with a grinding mill.

2.3 Extraction

2.3.1 Preparation of plant extract for larvicidal assay

The powdered stem bark (50 g) was repeatedly extracted (3 times) with 1 L of methanol with stirring at an interval of 4 h for 24 h. After filtration, the solvent was evaporated under reduced pressure in a rotary evaporator at 45ºC to afford the methanol crude extract (8 g).

2.3.2 Preparation of plant extract for antiplasmodial and cytotoxicity assays

The powdered stem bark (150 g) of *C. arereh* were extracted successively (3 times) with hexane (1.5 L), $CHCl_3$ (1.5 L), EtOAc (1.5 L) and MeOH (1.5 L) to give 1.5 g, 3 g, 3.5 g, and 12 g of extracts, respectively. Water extract was prepared by simple maceration of 100 g of powdered stem bark in 100 mL of distilled water maintained at ambient temperature for 4 h. Extract was filtered on filter paper and freeze-dried to yield 7.5 g.

2.4 Larvicidal Activity Assay

2.4.1 Collection and rearing of mosquitoes

Larvae of *Cx. quinquefasciatus* were collected in a beaker (1000 mL) from the natural ponds at the Botanical Garden, Faculty of Science, University of Khartoum and reared under laboratory condition. When the larvae were introduced into the lab, they were distributed in a number of aluminum dishes 10.6 inches wide and 1.6 inches deep containing dechlorinated tap water. Pupae and fourth instars larvae were removed to avoid any possible contamination of culture with pathogens. Only third instars larvae were used in the bioassays. Younger instars were provided with small amount of fine powdered-bread added daily until they reached the third instars stage. Water was changed every day to avoid scum formation; which might create toxicity. Eight hours of light were provided daily and rearing temperature was kept at 25±3ºC.

2.4.2 Preparation of test solution

Stock solution (2%, w/v) was prepared by adding 2 g of methanol extract of stem bark to 500 mL in dechlorinated tap water by sonication in an ultrasound bath for 20 min. The stock solution was then serially diluted by adding water to prepare the test concentrations required.

2.4.3 Bioactivity test

Larvicidal activity of the extract was determined by following the WHO standard procedure [17]. Initially, multiple 10-fold concentrations were set up to establish a working range (the highest extract concentration used was 1000 ppm). A second set of exposure was then carried out to establish the LC_{25}, LC_{50}, LC_{90} and LC_{95}. A control group consisted of 0.03 mL of methanol and 99.97 mL of distilled water and untreated sets of larvae in tap water were also run for comparison. Twenty-five laboratory reared 3rd instars larvae were transferred by means of dropper to the small test cups (250 mL), each containing 200 mL of the test solution. Each concentration together with the untreated control groups was replicated 4 times. Mortality was recorded after 24 hrs of exposure during which no food was offered to the larvae. Larvae of mosquito were considered dead when they float on the surface horizontally or settle on the bottom without moving.

The percentage mortality was calculated by using formula (1), and corrections for mortality when necessary were done using Abbot's [18] formula (2)

Mortality (%) = Number of dead larvae/ Number of larvae introduced X 100 (1)
Corrected mortality (%) = 1 - n in T after treatment/ n in C after treatment X 100 (2)

Where n = number of larvae, T = treated, C = control.

2.4.4 Statistical analysis

Data were subjected to probit analysis. The regression equation (Y= a+bx), lethal concentration that killed 25%, 50% 90% and 95% of the population (LC_{25}, LC_{50}, LC_{90} and LC_{95}), and regression coefficient (r2) were calculated.

2.5 Antiplasmodial Activity

The antiplasmodial activity of plant extracts was assessed on the chloroquine-resistant strain W2 strain of *Plasmodium falciparum* maintained in continuous culture according to the methodology described by Tragger and Jensen [19]. *P. falciparum* strains were cultivated using A^+ human erythrocytes at 50% haematocrit in RPMI 1640 medium supplemented with HEPES, $NaHCO_3$, 10% A^+ human serum and neomycin.

Plant extracts were dissolved in DMSO and incorporated in triplicate cultures. Negative controls treated by solvent (DMSO) and positive controls containing a range of chloroquine concentrations (2 to 100 µg/ mL) were added to each set of experiments.
The assays were performed in flat bottom, 96-well tissue culture plates containing 200 µL of culture medium. The asynchronous parasite cultures exhibited an initial parasitaemia of 1% and a haematocrit of 4%. Parasitaemia was evaluated after 48 h without medium replacement, by a flow cytometric technique derived from the method of Azas et al. [20] using the vital dye hydroethidine (HE, Interchim, Montluc¸on, France) that is converted by

metabolically active cells into ethidium. The interaction of ethidium and nucleic acids of parasites allows a distinction between infected erythrocytes and uninfected erythrocytes using flow cytometry. A stock solution of HE (10 mg/mL) in DMSO was prepared and stored at 20ºC. The culture medium was removed from each well of parasite culture plates. Two hundred microlitres of HE diluted 1/200 in phosphate-buffered saline (PBS) was added to each well and incubated for 20 min at 37ºC in the dark. The cells were then washed twice with PBS by centrifugation at 1200 rpm for 5 min and resuspended in a final volume of 1 mL of PBS in the tubes for fluorescence-activated cell sorter (FACS) analysis. The detectors of forward, side scatter and Fluorescence 2 (FL2) were set in logarithmic mode and 10000 cells were used for data acquisition. Both infected and uninfected erythrocytes were gated in the analysis and the percentage of parasitaemia (number of infected erythrocytes/total erythrocytes) x 100 was determined using the LYSIS II program (Becton-Dickinson).

2.6 Cytotoxic Activity

Cytotoxicity was assessed on chemosensitive subline K562 derived from a chronic myeloid leukemia and purchased from Dr J. Boutonnat (UMR-CNRS 5525, Université Joseph Fourier, La Tronche, France) [21]. Late log-phase K562 cells were incubated in RPMI 1640 (without phenol red) supplemented with 10% foetal calf serum, 2% l-glutamine and 1% penicillin-streptomycin mix (complete RPMI medium) and a range of compound concentrations incorporated in duplicate (final DMSO concentration less than 0.5%). Appropriate controls treated with or without solvent (DMSO), and various concentrations of doxorubicin (positive control), chloroquine and doxycycline were added to each set of experiments. After 72 h incubation at 37ºC and 6% CO_2, cell growth was measured by flow cytometry after staining monocytes with 5 lL of propidium iodide. Antiproliferative activity was evaluated by counting the number of live cells in a 100 lL sample. Inhibitory concentration 50% (IC_{50} K562) was defined as the concentration of drug required to induce a 50% in the K562 cell proliferation compared to the control. IC_{50} was calculated by non-linear regression analysis processed on dose–response curves, using the Table Curve software 2D v.5.0. IC_{50} values represent the mean value calculated from three independent experiments.

2.7 MTT Assay

The evaluation of the tested extracts cytotoxicity on the HepG2 cell line was done according to the method of Mosmann [22] with slight modifications. Briefly, cells in 100 lL of complete medium, (RPMI supplemented with 10% foetal bovine serum, 1% L- glutamine (200 mM) and penicillin (100 U/mL)/streptomycin (100 lg/ mL)) were inoculated into each well of 96-well plates and incubated at 37ºC in a humidified 6% CO_2 with 95% air atmosphere. After 24 h incubation, 100 lL of medium with various product concentrations was added and the plates were incubated for 72 h. At the end of the treatment and incubation, the medium was aspirated from the wells and 10 lL MTT solution (5 mg MTT/mL in PBS) was added to each well with 100 lL of medium without foetal calf serum. Cells were incubated for 2 h at 37ºC to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After this time, the MTT solution was aspirated and DMSO (100 lL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength with a microplate spectrophotometer. DMSO was used as blank and doxorubicine as positive control. Cell viability was calculated as percentage of control (cells incubated without extract). The 50% cytotoxic concentration was determined from the dose–response curve.

2.8 Phytochemical Screening

The powdered stem bark was subjected to phytochemical screening for the identification of major groups of chemical constituents using standard procedures [23,24].

3. RESULTS AND DISCUSSION

3.1 Larvicidal Property

The larvicidal activities of different concentrations of *C. arereh* stem bark methanolic extract after 24 h are represented in Table 1. Analysis of variance showed that there were highly significant differences among the thirteen treatments in their effects on *Cx. quinqucfasciatus*. No mortality was recorded on the water control, the control methanol and the lowest concentration (0.05 g/L) of *C. arereh* extract. The high range of 0.7—1.0 g/L concentration of extract resulted in level of mortality above 25%. The highest mortality percentage (40.2%) was caused by the 1.0 g/L concentration, which was significantly greater than all other values. It was followed by 35% mortality recorded for 0.9 mg/L concentration of *C. arereh* extract. Application in the range 0.4—0.6 g/L caused a morality in the range of 13—22.5%. On the other hand, a morality percentage which was below 10% was recorded for *C. arereh* extract of 0.3 g/L or less.

A positive relationship between the level of mortality and the concentration of *C. arereh* extract applied was observed. The percentage mortality was increasing with the increase in the concentration of *C. arereh* stem bark extract. A similar conclusion was reached when *Ferronia elephantum* leaf extract was used against *Cx. quinquefasciatus* [25].

Table 1. Larval mortality of the third larval instars of *Culex quinquefasciatus* exposed for 24 hours to different concentrations of *Cassia arereh* stem bark methanolic extract

Concentration (g/L)	Larval mortality (%)
Control(methanol)	0.0 ± 0.0
Control(water)	0.0 ± 0.0
0.05	0.0 ± 0.0
0.1	1.3 ± 0.5
0.2	2.5 ± 1.3
0.3	9.3 ± 2.1
0.4	13.1 ± 1.5
0.5	18.5 ± 1.9
0.6	22.5 ± 2.4
0.7	26.8 ± 2.8
0.8	31.5 ± 3.1
0.9	35.0 ± 4.2
1	40.3 ± 1.5
LSD	2.49

From the results of the test of the larvicidal activity of the different concentrations of *C. arereh* stem bark extract, the amount of the lethal dose was calculated. The results (Table 2) showed that the LD_{50} of *C. arereh* stem bark extract was 8230 ppm (8 g/L). However, approximately double this amount was required to reach LD_{75} (16007 ppm). On the other

hand, five times the amount of LD_{50} was required to reach LD_{95} (40680 ppm) (40.68 g/L). The study result obtained was comparable with earlier reports. The toxicity to the late third instars larvae of *Cx. quinquefasciatus* by methanolic leaf extract of *Benincasa cerifera* showed LD_{50} values of 1189.30 ppm [26]. Also, the leaf extracts of *Acacia ferrugginea* showed larval mortality at LC_{50} of 5362.6 ppm against the third larval instar of *Cx. quinquefasciatus* after 24 h treatment [25].

Table 2. Lethal dose of *Cassia arereh* stem bark methanolic extract against *Culex quinquefasciatus*

LD_{25} (ppm)	LD_{50} (ppm)	LD_{75} (ppm)	LD_{90}(ppm)	LD_{95} (ppm)	Regression equation
4235	8230	16007	29325	40680	Y=0.556+2.321X

3.2 Antiplasmodial Activity

A preliminary screening of the chloroform, ethyl acetate, methanol and aqueous extracts of *C. arereh* against the W2 strain was carried out. Results are presented in Table 3. The *in vitro* antiplasmodial inhibitory concentrations 50% (IC_{50}) of the crude extracts of ranged from 12.5 to > 50 µg/mL. The highest antiplasmodial activity was revealed by the $CHCl_3$ extract followed by the MeOH and EtOAc extracts with IC_{50} values of > 12.5, > 25 and 27.8 µg/mL respectively. The H_2O extract were not effective *in vitro* against *P. falciparum* (IC_{50} > 50 µg/mL). Water is the extraction solvent used the most (and often the only one available) in traditional medicine in endemic areas. However, even if only extracts with organic solvents were active *in vitro* against *Plasmodium*, it would be very interesting to carry out further investigations on the biological effects of water extract during antimalarial treatment. Indeed, some plants (or parts of plants), without direct activity against the parasite, can show properties that could be acting on the symptoms of malaria (fever, anemia, hypoglycemia etc.), and/or increase the bio-availability and/or enhance immunological stimulation *in vivo* [27]. Another explanation is that the water extract could contain prodrugs non-active by themselves [10]. In this case, these precursors of the active compounds have to be metabolized *in vivo* into active antimalarials by the identification of the mechanisms of their *in vivo* activation. Moreover, the periods of the year of plant collection are known to play an important role in the variation of the type of compounds found in plants as well as their concentrations [10].

Table 3. Antiplasmodial activity and human cell toxicity of *Cassia arereh* stem bark extracts

Extract/drug	W2 Antiplasmodial	Human cell toxicity	
	Activity IC_{50} (µg/ mL)	K562S IC_{50} (µg/ mL)	HepG2 IC_{50} (µg/ mL)
Chloroform	> 12,5	> 12,5	> 10
Ethyl acetate	27.8	> 50	> 20
Methanol	> 25	> 25	> 10
Water extract	> 50	> 50	> 20
Chloroquine	0.36	15.3	15.4
Doxycycline	3.34	7.6	10.3
Doxorubicine		0.006	0.2

Chloroquine and doxycycline were used as antiplasmodial drug compounds of reference. Doxorubicin was used as a drug compound of reference for human cell toxicity.

3.3 Cytotoxicty

The *in vitro* cytotoxicity on 2 reference cell-lines (K562S and HepG2) of the organic solvent extracts showed IC_{50} values ranging from > 10 to > 50 µg/mL superior to that of Doxycycline and comparable to Chloroquin. The water extract was not toxic to both cell-lines suggesting to some extent the safety of this plant in the traditional medicine. Furthermore, this low *in vitro* cytotoxicity is confirmed by the frequent use of *C. arereh* stem bark extract in indigenous medical systems for a considerable time in the Sudan with no evidence of clinical toxicity while ethnobotany surveys also exclude pronounced human toxicity.

3.4 Phytochemical Screening

The presence of anthraquinones, terpenes, sterols and flavonoids were reported from various *Cassia* spp [28,29]. Results of phytochemical screening indicated that the stem bark of *C. arereh* contained chemical components like anthraquinones, flavanoids, terpenes, steroles and tannins. Okwute [30] reported that terpenes, alkaloids, saponins, phenolic compounds and cardiac glycosides are known to possess antimicrobial and antiplasmodial activity and pesticide properties. This confirms the importance of the stem bark in future studies of this plant in the search for the molecules responsible for these activities. Osman et al [31] reported the antiplasmodial activity of anthraquinones, thus isolation and characterization of compounds from the $CHCl_3$ extract may offer interesting bioactive molecules.

4. CONCLUSION

In conclusion, this is the first report on the *in vitro* larvicidal, antiplasmodial and cytotoxicity properties of the stem bark of *C. arereh*. More *in vivo* and *in vitro* studies along with detailed phytochemical investigation are needed to examine the beneficial use of *C. arereh* in the prevention and therapies of some diseases.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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