RESEARCH ARTICLE

In-vitro Anti-cercarial activity of extracts and steroidal alkaloids from the stem bark of *Holarrhena floribunda* (G. Don) Dur. & Schinz

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Abstract

Schistosomiasis continues to be the leading cause of morbidity and mortality among the neglected tropical diseases. Apart from the high cost of chemotherapy, concerns over drug resistance and tolerance have been raised in the past decade**.** The aim of the study was to evaluate the anticercarial activity of extracts and compounds from the stem bark of *Holarrhena floribunda* on cercaria of Schistosoma haematobium. Hydroethanolic and alkaloidal extracts from the stem bark of *H. floribunda* were tested on cercaria at concentrations between 500.00 and 15.625 μg/mL for 180 minutes and assessing the percentage viability at time intervals of 0, 15, 30, 60, 120 and 180 minutes. Praziquantel, used as reference drug, and the isolated compounds were tested at similar concentrations. The cercaria mortalities and IC_{50} of extracts and compounds were estimated after 30 minutes of incubation. The 70 %v/v ethanol extract showed the highest activity (IC_{50}=20.09$\pm$1.11 μg/mL) with praziquantel giving IC_{50} of 695.50$\pm$1.12. The alkaloids holonamine, holadienine and conessine, isolated from the stem bark, showed considerable cercaricidal activity with the latter recording an IC_{50} of 33.28$\pm$1.04. The study gives first-hand knowledge of the anti-cercarial activity of *H. floribunda* and its steroidal alkaloids. This gives credence to the traditional uses of the plant as an anti-parasitic agent.

Keywords: Cercaria; schistosomiasis; conessine; praziquantel; Schistosoma haematobium 1

Introduction

Among the neglected tropical diseases, Schistosomiasis ranks second in terms of the number of deaths it causes. [1] It is caused by the infective stage (cercariae) of Schistosoma species. In Africa, the species *S. mansoni, S. haematobium, S. guineensis* and *S. intercalatum* are mostly implicated in the disease although *S. japonicum* and *S. mekongi* also cause schistosomiasis in man. [1] In 2001, it was estimated that *S. haematobium* and *S. mansoni* accounted for 166 million cases of schistosomiasis. [2] Death from *S. mansoni* and *S. haematobium* infection is estimated to be around 130,000 to 150,000 annually through complications such as kidney failure and portal hypertension. [1]

In Ghana, Aryeetey et al. [3] estimated a prevalence of 54.8%-60% after sampling 2,562 members in eight villages in the Ga West and Akuapem North municipality, endemic to *S. haematobium*. However, recent studies done in villages along the Tono irrigation canal in the Upper East Region of Ghana, estimates a prevalence of 33.2% and 19.8% respectively for *S.*

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haematobium and *S. mansoni*, with a combined prevalence of 47.7%. [4]

With the wide geographical distribution of schistosomiasis, it has become increasingly difficult to treat. [1] Until 1972, the WHO had not accepted any drug for population-based chemotherapy although some drugs were in use. [5] Praziquantel (PZQ) remains the drug of choice although there have been fears of resistance as well as its inability to prevent reinfection and kill juvenile schistosomes. Artemether (ART) is known to be effective in killing juvenile stages of schistosomes. In endemic countries like Ghana, with high incidence of malaria, other alternatives to ART is needed, as resistance of plasmodium parasites to ART can further dampen treatment options for malaria. [6] It is therefore not surprising that combination therapy of PZQ and ART as a means of treatment was discontinued. There is the need to look for alternate effective, cheaper and more available sources of drugs to delay tolerance and resistance and to serve as a source of novel agents for the treatment of schistosomiasis in endemic regions of Africa and Ghana where access to quality health care is a challenge. A number of novel drugs have found their source from plants. Investigation of the efficacy of medicinal plants used in traditional medicine for the treatment of diseases led to the discovery of a number of drugs used in conventional medicine. *Holarrhena floribunda* has been reported to have considerable therapeutic properties. [7] The plant is native to West Africa and is known in Ghana as 'osese' (among the Akans). It is an antifungal, antibacterial and antidiabetic agent [8–10] and also finds use traditionally for the treatment and management of malaria, fever and female sterility [8–10].

Studies have shown the leaf and stem bark extracts of *Holarrhena floribunda* and its alkaloid fractions to show significant in vitro activity against bloodstream forms of Trypanosoma brucei rhodesiense. [11] Yemoa et al. [12] demonstrated the antimycobacterium activity of extracts and alkaloids from the aerial part of *H. floribunda* against Mycobacterium ulcerans. Thus, the potential of *H. floribunda* in the management of the neglected tropical diseases is not in doubt. Therefore, in this study, *H. floribunda* extracts fractions and isolated compounds were evaluated against aquatic forms of the parasite (cercaria from Bulinus species) as a means of paving the way for the development of cercaricidal agents for water treatment in endemic areas.

Materials and methods

Solvents and chemicals

The solvents and reagents were of analytical grade. Ethanol, petroleum ether, ethyl acetate, methanol, acetic acid, concentrated ammonia, dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich company (Darmstadt, Germany), tween

80 (Nacalai Tesque (Kyoto, Japan), desk lamp, sterile petri dish, pyrex beakers, 24-microtiter well plate, conditioned water (Lab Aid, Accra, Ghana), Leica microscope, counting chamber (GmbH, Wetzlar, Germany). Silica gel 60 (70-230 Mesh) and precoated silica gel TLC plate F_{254} obtained from Merck Millipore (United Kingdom).

Equipment

These include rotary evaporator (Buchi, Switzerland), LC-MS (LTQ Orbitrap spectrometer, Thermofisher, USA) coupled to Agilent 1200 (Santa Clara, USA) HPLC, NMR techniques were performed on Bruker Avance DRX-500 (500 MHz), Varian Unity Inova (500 MHz), Inova spectrometer (600 MHz) or AVANCE III HDX (Bruker BioSpin GmbH; 700 MHz), Jenway UV-VIS spectrophotometer, Heraeus Biofuge Primo Centrifuge, Bruker Fourier transform infrared (FT-IR) spectrometer, Stuart SMP10 digital melting point apparatus (Cole-Palmer, Staffordshire, UK).

Plant collection and identification

The stem bark of *H. floribunda* was harvested from Axim (4°52'19.3"N, 2°14'32.3"W) in the Western region of Ghana in July 2017. It was identified and authenticated by Mr Emmanuel Agyarkwah of the Herbarium section of the University of Cape Coast and given a voucher number 1761.

Material processing and extraction

The stem bark of *H. floribunda* was rid of extraneous matter and dried at room temperature (25 -27 ºC) for one week. It was pulverized and 1500 g of the powdered bark extracted with 5 L of 70% v/v ethanol by cold maceration. It was then filtered with Watman number 3 filter paper and concentrated using a rotary evaporator. The filtrate was dried on a bath at a temperature of 70 °C to obtain the crude 70% v/v ethanol extract (HYD; yield 12% $^{w}/_{w}$). Part of the extract (10 g) was kept in a desiccator until required for use and the remainder (170 g) subjected to alkaloidal extraction according to the method described by Shah and Seth, (2012). Briefly, the 70% v/v ethanol extract (170 g) was digested with 10% glacial acetic acid (pH=4.9) and basified with dilute ammonia solution to a final pH of 8.8. The solution was then partitioned with 2 L of chloroform in successive 200 mL aliquots. The combined chloroform extract was concentrated and dried to afford a total alkaloidal mixture (ALK) of weight 51 g.

Column fractionation of Alkaloid mixture

The alkaloidal mixture (50 g) was loaded onto a glass column (60 cm × 3 cm) packed with silica gel (70-230 mesh).

It was then eluted with mixtures of petroleum ether, ethyl acetate and methanol of increasing gradient polarity starting with 100% pet-ether to 100% ethyl acetate and subsequently to 100% methanol. Fifty-six fractions were collected in 60 mL aliquots and bulked into six sub-fractions based on their TLC profiles (coded CF 1-6). Active fraction CF1 (9.5 g) was further column chromatographed on silica gel (70-230 mesh) using gradient mixtures of pet ether, ethyl acetate and methanol. Sixty-three fractions were collected in 20 mL aliquots and bulked into six sub-fractions which were labelled as Hf1, Hf2, Hf3, Hf4, Hf5 and Hf6.

HPLC separation of compounds in active fraction Hf1

Fraction Hf1 (2.15 g) was subjected to purification by preparative HPLC [MeOH-H_2O + 0.1% HCOOH 98:2, 2 mLmin^{-1}] to yield compounds Hfa, Hfb and Hfc. High performance liquid chromatography (HPLC) was performed on a Nexera Shimadzu LC using a Phenomenex Gemini C18 column (10 × 250 mm, 10 μm particle size) and a chromeleon software system. The set up consisted of a gynkotek pump equipped with a Dionex DG-1210 geasser, a Dionex UVD 340S detector and a Dionex Gina 50 auto-sampler. During the separation, MeOH-H_2O solvent system was employed for travelling the RP column with 0.1% formic acid (HCOOH) as buffer. The gradient of 95% methanol 5 % water to 100 % water was employed in the run. Flow rate was maintained at 0.62 mL/minute with 25ºC column temperature.

Experimental

Liquid Chromatography-Mass Spectrometry (LC-MS

LC-MS was employed to check the purity and provide the exact mass and molecular formula of the isolated compounds. Mass fragmentation ($MS^{2/3}$) experiments were performed by collision-induced dissociation (CID) to evaluate the structural features of compounds based on the fragment information. The HPLC-MS experiments were carried out on a LTQ Orbitrap spectrometer (Thermofisher, USA) equipped with a HESI-II source. The spectrometer is equipped with an Agilent 1200 (Santa Clara, USA) HPLC system consisting of a pump, PDA detector, column oven (30 °C) and an auto sampler. The spectrometer was operated with nominal mass resolving power of 60 000 at m/z 400 with a scan rate of I Hz to afford high-accuracy mass measurements within 2ppm deviation. The HPLC separations were performed using a Luna C 18 column 10 x 250 mm, 10 μm particle size (Phenomenex, Torrance, USA) with H_2O (+ 0.1% HCOOH) (A) / MeOH (+ 0.1% HCOOH (B), gradient flow rate 0.4mL/min. Samples were analysed by using programme as follows; linear gradient from 5% A to 100% B over 26 min, 100% B isocratic for 5 min, then after the system returned to

its initial condition (95% A, 5 % B) within 0.5 min and was equilibrated for 4.5 min. The experiments were run in positive mode under the following parameters: nitrogen sheath gas flow (arbitrary units) 55, nitrogen auxiliary gas flow (arbitrary units) 8, spray voltage 5 kV vaporizer temperature 400 °C, capillary temperature 300 °C, capillary voltage 20 V and tube lens 100 V. MS/MS experiments were achieved by collision-induced dissociation (CID, 35 ev) in the Orbitrap mass analyser. The collision gas used was Argon. The LC-HRMS data was analysed through the software ThermoXcalibur 2.2 SP1.48 (Thermo Fischer Scientific Inc., USA).

Nuclear Magnetic Resonance Spectroscopy (NMR

One dimensional (1D) NMR spectroscopic techniques including ^1H and ^{13}C-NMR, together with two-dimensional (2D) NMR spectroscopic techniques such as correlation spectroscopy (COSY), hetero-nuclear single quantum correlation (HSQC), hetero-nuclear multiple bond correlation (HMBC) and nuclear over-hauser enhancement spectroscopy (NOESY) were used for the structural elucidation of compounds. 1D NMR, COSY, HSQC and HMBC afforded the detailed information which was used to assign the planar structure of the compounds. Solvents used for NMR analysis were deuterated chloroform $CDCl_3$ and methanol-d_4 (CD_3OD) deuteron GmbH, Kastellaun, Germany). The pure compounds were dissolved and transferred into an NMR tube (5 x 203 mm) for measurements. NMR spectra were recorded at 25 °C on a Bruker Avance DRX-500 (500 MHz), Varian Unity Inova (500 MHz), Inova spectrometer (600 MHz) or AVANCE III HDX (Bruker BioSpin GmbH; 700 MHz). Chemical shifts were quoted in parts per million (ppm) relative to the chemical shift of the internal standard tetramethylsilane (d 0 ppm)

In Vitro studies with *S haematobium*

Harvesting and maintenance of snails

Naturally infected snails, Bulinus species, were collected from endemic areas in their natural habitat, Tomefa near Weija in the Greater Accra Region of Ghana. Identification of Bulinus species was done according to the method described by Brown et al [13]. The snails were then transported in well ventilated aquarium bowl (measuring 27 cm x 12 cm) to the University of Cape Coast Biomedical Science laboratory, where the work was done. The water in the aquarium (dechlorinated) was changed thrice a week to get rid of faeces and foul smell due to feeding. Snails were allowed five days to acclimatize to the new environment and fed on lettuce.

Shedding of Cercariae

The procedure described by Tucker et al. [14] with extensive modification was followed. Snails numbering 10 were placed in a 10 mL test tube with 2 mL of conditioned water (5 snails per mL of conditioned water). The tubes were placed on a rack and then exposed to early morning sunlight (8 am-10 am) for 20 min, then, placed under desk lamps with fluorescent bulb (60W) light source for another 40 min. The snails were then removed from the test tubes with the aid of forceps and placed in conditioned water temporarily before being returned to the aquarium.

Contents of the test tubes were filtered and then poured into a petri dish. A Pasteur pipette was used to measure an aliquot of 1 mL from the petri dish and dispensed into counting wells. An inverted microscope with objective lens (4×) was used to estimate the average number of cercariae. Wells with significant cercariae (18-22) were standardised to obtain an average of 20 cercariae per well.

Preparation of solutions of extracts and isolates

Stock extract and total alkaloidal extract concentration of 1000 μg/mL was prepared and then serially diluted (two fold) using distilled water to obtain concentrations 500.0 μg/mL, 250.0 μg/mL, 125.0 μg/mL, 62.5 μg/mL, 31.25 μg/mL and 15.625 μg/mL. The isolated compounds Hfa, Hfb and Hfc as well as the reference drug praziquantel were prepared in a similar way.

Cercariacidal activity

The procedure described by Tekwu et al [15] was followed with little modification. An estimated freshly shed 20 cercariae, was placed in each well of the 24-microtiter plate with equal volume of solution. and the extracts and compounds tested at six concentrations of 500-15.625 μg/mL (prepared by two fold dilution). Survival and mortality was observed at 0, 15, 30, 60, 120 and 180 min. Dead cercaria are non-motile, loose their tail and sink to the bottom of the well. Percentage viability: dead cercaria and those alive were observed and calculated at different times (0, 15, 30, 60, 120 and 180 min). All tests were carried out in duplicates. Negative controls were prepared with 100% unconditioned water and 1% DMSO. Praziquantel, at similar concentrations, was used as the positive control. IC$_{50}$ were calculated at 30 min for all extracts and compounds.

Percentage inhibition

$Percentage\ (\%)\ viability\ was\ calculated\ as = \frac{\{(K) - (DC)\}}{K} \times 100$

Where K is total number of cercariae at the beginning of the experiment

DC is dead cercariae at the specific times (0, 15, 30, 60, 120 and 180 min)

Statistical analysis

Graphpad ® Prism for Windows Version 7.0 (Graphpad Software, San Diego, CA, USA, 2017) was used for all statistical analysis. The concentration-response curves after thirty minutes of incubation with the plant extracts and compounds was then used to determine the concentration at which 50% of the cercaria lost their viability (i.e. IC$_{50}$). The IC$_{50}$s were determined by plotting a nonlinear regression curve (log concentration of inhibitor verses % viability).

Results

Chromatographic separation of the hydroethanolic stem bark extract of *H. floribunda* resulted in the isolation of three compounds. The characterization of compounds was done by High Resolution Electron Spray Ionisation Mass Spectrometer (HR-ESIMS) and nuclear magnetic resonance (NMR) analysis. SciFinder (CAS, a division of the American Chemical Society) search of the plant was employed in the identification of isolated compounds. All data obtained was also compared with published literature on related compounds.

Identification of compounds

Compound HF1C was isolated as a yellow crystal, soluble in methanol with a melting point of 125-127°C (literature: 126-127 °C). Its ^{1}H NMR spectrum exhibited three singlets at δ 0.95, 2.23, 2.33 and a doublet at δ 1.07 characteristic of five methyl groups. An olefinic proton occurred at δ 5.32 and the aliphatic protons resonated between δ 0.92 and 2.44. The two protons on C-18 are diastereotopic, one of which appeared at δ 3.0 (H-18') and the other at δ 1.89 (H-18"). Furthermore, it contains a β-CH$_3$ on C-10, trans-fused B and C rings, C and D rings. The ^{1}H NMR signals of two methyl groups (δ 2.21, 6H, s) suggested the presence of N(CH3)$_2$ group on C-3 (Figure 1). Signals for all 24 carbon atoms appeared in the ^{13} C-NMR spectrum. In NOESY, H-3 (δ 2.25) had correlation with H-1β (δ 1.99) which also had correlation with H-19 (δ 0.89). This result confirmed the α-N(CH$_3$)$_2$ group on C-3. The β-CH$_3$ on C-20 was also confirmed by the correlation between H-21 (δ 1.00) and H-16β (δ 1.58). Based on the correlation between H-22 (δ 2.15) and H-21, a β-CH$_3$ on position 22 was identified, due to the formation of sp3 type of the nitrogen atom in the E ring. Based on the NMR data, compound HF-3 was suggested to be a steroidal alkaloid with an unsaturated bond in the skeleton (Figure 1a). It has a double bond equivalent of 6 which is accounted for by the five rings and one double bond present in

ring B (Figure 1). The molecular formula of HF1C, $C_{24}H_{40}N_2$ determined by HRESI- MS/MS (m/z): 357.3267 [M + H]$^+$; calculated for $C_{24}H_{41}N_2{}^+$: 357.3270. Fragments appearing in the MS/MS spectra of Hf1C, showed a [M-C_2H_7N+H] $^+$ peak at m/z 312.269, then [-C_2H_6] $^+$ peak at m/z 206.190, and [-C_2H_3] $^+$peak at m/z 283.23 and a fragment at m/z 268.229. The NMR and mass spectra data agreed with that published for conessine [11] which has been reported in the bark of this plant. [16] It has three six-membered rings of which ring B adopts a half-chair-like conformation while rings A and C forms a chair-like one. The cyclopentane ring D shows a half-chair conformation and the methylpyrrolidine ring E adopts an envelope conformation. The dimethylamino substituent in ring A is equatorially oriented. [17]

Similarly, compound HF1B was isolated as a yellow amorphous powder soluble in methanol. The ^{1}H NMR spectrum exhibited two tertiary methyl groups at δ 2.29 (N-Me) and 1.19 (H-19) and a secondary methyl group at δ 1.08 (d, J=6.36 Hz, H-21)(Figure 1b). The chemical shifts of N-Me and H-21 were comparable with those of conessine derivatives. [18] The presence of a conjugated ketone was suggested by the three deshielded olefinic protons at δ 7.03 (d, J=10.14 Hz), δ 6.21 (dd, J= 10.14, 1.91Hz) and δ 6.06 (t, J = 1.91 Hz) which were assigned to protons H-l, H-2 and H-4, respectively (Figure 1b). From the mass spectrum of $C_{22}H_{31}NO$ (HRMS, [M+1] 326.248) calculated 326.49, fragments appearing in the MS/MS spectra of Hf1b, showed a [M-Me] $^+$ peak at m/z 311.225), [M-C_2H_6]$^+$, peak at m/z 296.201, [M-Me, CO]$^+$,peak at m/z 283.23 and a fragment at m/z 268.229.

HF1B has a pentacyclic steroidal nucleus (Figure 1b); the ring junctions share the same stereochemistry reported for steroidal alkaloids. In addition, the number of double bond equivalents 8 is accounted for by the 5 rings and 3 double bonds. These data characterized HF1B as holadienine, which was corroborated by ^{13}C NMR and HMBC experiments as well as various fragments in the mass spectrum, characteristic of this type of compound. [19] Holadienine was previously isolated from the stem bark of *H. pubescens.* [20]

Compound HF1A was also isolated as a white amorphous powder soluble in chloroform. The ^{1}H and 13C NMR spectra was consistent with that of holadienine (compound HF1B) except for the presence of a hydroxyl group at C-11 and C=N group at position 18 (Figure 1c). The presence of the hydroxyl group resulted in C-11 of HF1A resonating more downfield at δ 68.91 compared to δ 25.1 in holadienine. Similarly, the double bond at C-18 (of HF1A) resulted in the downfield signal of δ 168.37 with that of holonamine resonating at δ 68.91 (no double bond). Signals belonging to all the 21 carbon resonances were accounted

for with the molecular mass established as $C_{21}H_{27}NO_2$ (HRMS, [M+1] 326.20) calculated 326.2120. HF1A has a pentacyclic steroidal nucleus; the ring junctions share the same stereochemistry reported for this class of compounds. It has five rings and four double bonds in the structure accounting for the 9 double bond equivalents. The ^{13}C NMR and complete ^{1}HNMR spectral assignments have also been made. These data characterized HF1A as holonamine which agreed with that published for this compound. [11]

Anti cercarial activity

The present study focussed on the anti-cercarial activity of extracts (total hydroethanolic extract and its alkaloidal fraction) and steroidal alkaloids isolated from *H. floribunda* on *S. haematobium cercariae*. The extracts showed concentration and time-dependent cercariacidal activity whereas all the normal control cercaria survived throughout the entire duration of incubation. Praziquantel, the reference drug used in the management of adult worms of Schistosoma, could not achieve 0% viability at all the tested concentrations throughout the 180 minutes of the test. Its maximum effect was 50% mortality of cercariae which occurred at its highest concentration of 500 μg/mL (Figure 2a).

The alkaloidal fraction (ALK) showed 100% mortality at all concentrations within an hour (Figure 2b). This was similar to that of the crude 70% v/v ethanol extract (HYD) except that its lowest concentration of 15.65 μg/mL could not achieve 100% mortality of the cercaria (Figure 2c). Time-course curves of the isolated compounds holonamine (Hf1A), holadienine (Hf1B) and conessine (Hf1C) showed a similar concentration and time-dependent effect except that the extracts showed higher cercariacidal effect at all concentrations than the compounds. The least effective cercaricidal agents among the three compounds was Holadienine (Hf1B) as its highest concentration of 500 μg/mL was able to achieve 100% mortality of the cercaria at the 180 min of incubation (Figure 3b). Conessine (Hf1C) showed a remarkable cercariacidal effect as concentrations up to 62.50 μg/mL showed 100% mortality of the cercaria within just 15 min of incubation (Figure 3c). On the other hand, concentrations up to 31.25 μg/mL of holonamine (Hf1A) achieved 100% cercariacidal effect within 120 min of incubation (Figure 3a)

Concentration-response curves (Figure 4) were used to establish their potencies in terms of the IC$_{50}$ (Table 1). The total crude extract (HYD) exhibited the highest cercaricidal potency with an IC$_{50}$ value of 20.09±1.11 μg/mL which was about 34 times more potent than the reference drug, Praziquantel. This was followed by the conessine, total alkaloidal fraction, holonamine, and holadienine (Table 1).
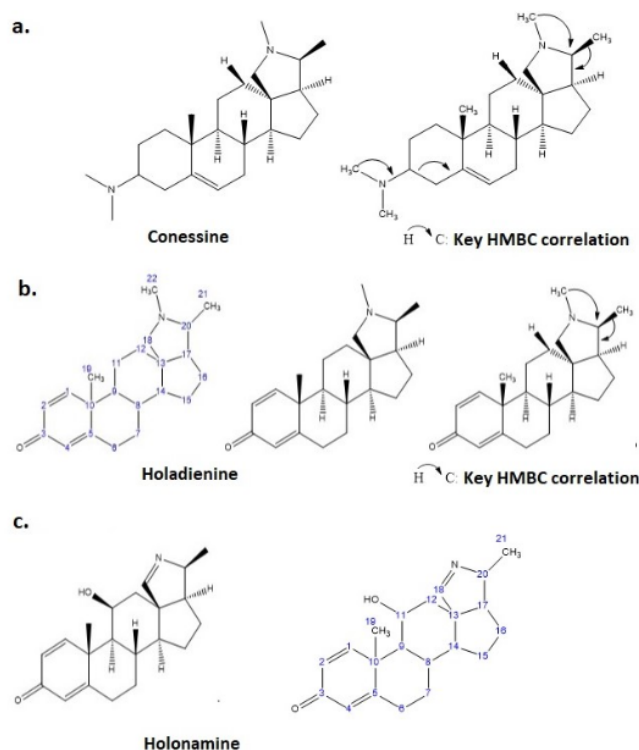
Figure 1 Structures of conessine, holadienine and holonamine showing key HMBC correlations

**Table 1** Anti-cercarial activity of extracts and compounds of *H. floribunda*

Extracts/compounds	IC$_{50}$ (μg/mL)	Negative control % mortality
Praziquantel (PZQ)	695.50$\pm$1.12	0
Total crude (HYD)	20.09$\pm$1.11	0
Alkaloid extract (ALK)	53.20$\pm$1.33	0
Holonamine (HF1A)	53.24$\pm$1.28	0
Holadienine (HF1B)	470.80$\pm$1.00	0
Conessine (HF1C)	33.28$\pm$1.04	0

Discussion

Schistosomiasis, one of the neglected tropical diseases, is on record to have debilitating effect on about 20 million sufferers [1] with 218 million people requiring prophylaxis in endemic areas, to stay off the disease. [21] The rural poor in Africa, especially Sub-Saharan Africa, are the most affected. The drug of choice, praziquantel, has enjoyed a monopoly of use against adult schistosomes for over three decades. However, with the efficacy of antibiotics on the wane due to microbial resistance, legitimate concerns over a similar fate for praziquantel has been echoed and thus the search for alternative agents is an important research agenda. A call for an integrated approach to sustainable management of the disease involving mass chemotherapy, behavioral changes towards risk factors, control of snails and larval forms of the parasite has been made and rightly so.

In this study, the total hydroethanolic extract (HYD), its alkaloidal fraction as well as isolated steroidal alkaloids were tested in vitro against cercariae of S. haematobium using praziquantel as reference drug. HYD showed higher cercaricidal activity than its alkaloidal fraction and compounds (Table 1). The activity of the extract was about 34 times higher than that of praziquantel used as reference but only three times more potent than its alkaloidal fraction (Table 1). Thus purification resulted in loss of anti-cercarial activity.

Conventional agents or drugs for the control of cercaria are not widespread. A number of plant materials, such as Balanites aegyptiaca, have been used to control snails and cercaria. Thus the high cercaricidal activity exhibited by H. floribunda extract affords a novel treatment option to be considered in an integrated control of schistosomiasis involving water treatment in endemic
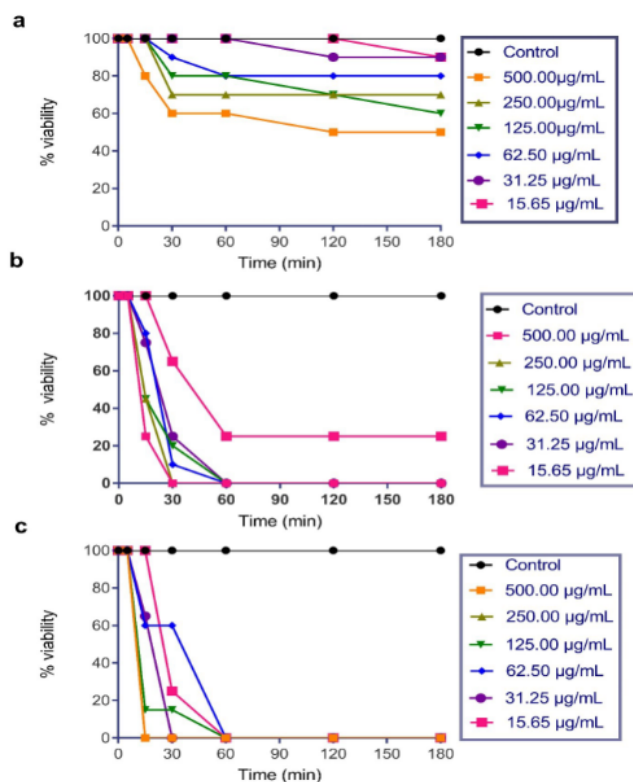
Figure 2 Time dependent percentage viability of *Schistosoma haematobium cercari*a exposed to **a**. Praziquantel (PZQ) **b**. crude extract (HYD) **c.** crude alkaloid extract (ALK) of *H. floribunda*

areas. Praziquantel is on record to be very active against adult schistosome worms, but inefficient against the younger stages of schistosomes such as cercariae, schistosomula, preadults, and juvenile adults. [15] This reflected in the low activity recorded in this study. As a result, repetition of treatment is sometimes necessary to kill those parasites that have since matured.

The alkaloidal fraction and its isolates also showed considerable activity. The alkaloid Conessine (Hf1C) showed anticercarial activity comparable to the hydroethanolic extract (Table 1). This was followed by holonamine (Hf1A) and holadienine (Hf1B). Holonamine, like the other compounds, exhibited a time dependent activity in relation to the percentage viability but deviated from the norm with regards to its linear relationship in terms of the concentrations (Figure 3a). Thus, alkaloids from H. floribunda may be responsible for it anti-cercarial activity. This is the first report of the cercaricidal activity of H. floribunda extracts and its alkaloids. Alkaloids from Holarrhena species have shown diverse biological activities including considerable anti-trypanosomal [11], antimicrobial and cholinesterase inhibitory activities. [22] H. floribunda, used by traditional healers in Benin to treat buruli ulcer, was reported to exhibit significant in vitro

antimycobacterial activities. [12] The steroidal alkaloids holaphyllinol, holamine, holaphyllamine and holadysamine were found to be responsible for the Mycobacterium ulcerans inhibitory effect with the latter exhibiting the highest activity. This study and previous work on Holarrhena species bring the genus into the spotlight as clear candidates in the search for novel drugs to mitigate microbes and parasites responsible for some of the neglected tropical diseases.

The anticercarial activity of the compounds conessine, holonamine and holadienine was lower than that of the total crude extract, suggesting a possible synergistic action of the steroidal alkaloids. The three compounds have a similar steroidal framework but the absence of the α-N(CH$_3$)$_2$ at C-3 in holonamine and holadienine appear to have resulted in the higher activity of conessine than the others. Conessine, isolated from the bark of *Holarrhena antidysentrica* exhibited substantial anti-malarial activity. [23] In another study, conessine showed antimicrobial activity against four Bacillus species. [24]

This research has highlighted the potential of hydroethanolic extract of *H. floribunda* or its active metabolite conessine for consideration in the new global strategy for the manage-
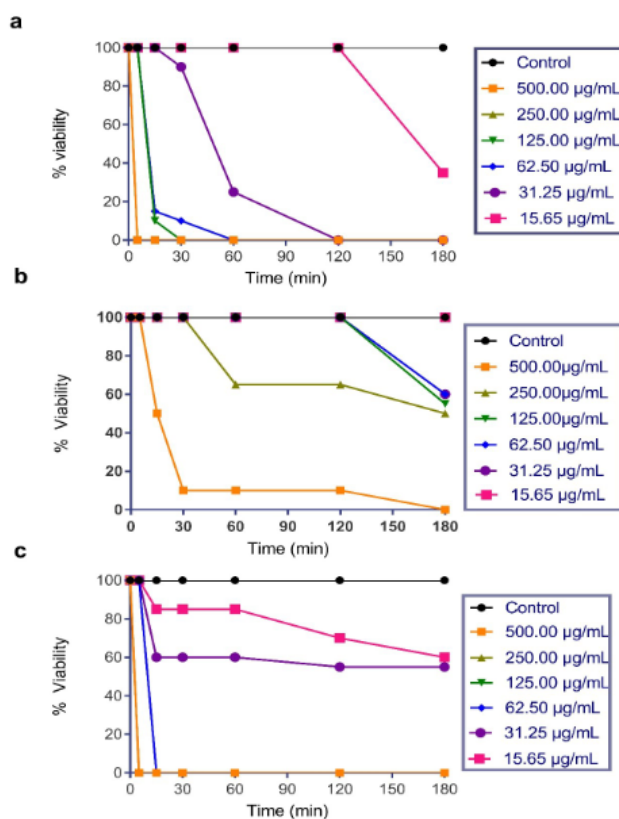
Figure 3 Time dependent percentage viability of *Schistosoma haematobium cercaria* exposed to compounds **a**. holonamine (Hf1A) **b**. Holadienine (Hf1B) and **c.** Conessine (Hf1C) isolated from *H. floribunda*
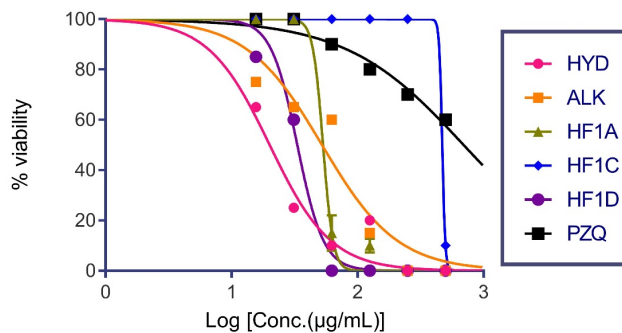
Figure 4 Concentration-response curves on the %viability of *S. haematobium cercaria* exposed to praziquantel (PZQ), crude extract (HYD) and alkaloid extract (ALK) of *H. floribunda* as well as holonamine (Hf1A), Holadienine (Hf1B) and Conessine (Hf1C) isolated from the plant.

ment of schistosomiasis which focuses on the two main transmission pathways in the schistosome life cycle: parasites' path from humans to snails, and their path from snails to humans. Treatment with PZQ acts only on the transmission pathway from humans to snails and only for as long as treatment is given. Some treatment approaches have harnessed the efficacy of Artemether (ART) (and other artemisinin derivatives), found to be effective against cercaria and juvenile schistosomes during the first 21 days of infection in both animals and humans. [25] Thus the combination of PZQ-ART is expected to improve cure rates, reducing the reservoir pool of infected individuals needed to halt transmission. [26] This is notably helpful due to the inability of PZQ to kill migrating schistosomula and the early stages of the disease. [27]

A multifaceted integrated approach complementing PZQ treatment with snail and cercaria control in water should be considered in which case *H. floribunda* extracts and its alkaloids are important leads.

Conclusion

The present study has shown that *H. floribunda* stem bark extract and steroidal alkaloids demonstrates anti-cercarial activity, *in vitro*, against cercariae of *S. haematobium*. This gives credence to the traditional use of the plant as an anti-parasitic agent.

Conflict of interest

We declare no conflict of interest

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