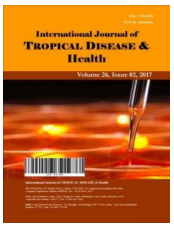

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In vitro Cercaricidal Activity of Fractions and Isolated Compounds of *Erythrophleum ivorense* (Fabaceae) Root Bark against *Schistosoma haematobium*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Introduction: *Schistosoma haematobium* is one of the species of Schistosoma responsible for schistosomiasis in humans, a major public health problem worldwide. Praziquantel, the most effective drug against all adult stages of human schistosomiasis, faces the threat of resistance and

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also has sub-optimal efficacy against cercaria, an immature form of schistosomiasis. This underscores the need to search for an alternative anti-schistosomal drug with pronounced activity particularly against cercaria.

Aim: This study investigated anti-cercarial activity of total crude (70% ethanolic extract), fractions (methanolic, ethyl acetate and petroleum ether) and isolated bioactive compounds from the root bark of *Erythrophleum ivorense.*

Study Design: *In vitro* anti-cercarial activity was evaluated using 20 freshly shed cercariae from *Schistosoma haematobium* species transferred into 20 well plates. Cercaricidal effect of the various concentrations (15.6, 31.3, 62.5, 125.0, 250.0 and 500.0 µg/mL) of test extracts and compounds were observed for 3 hours using an inverted microscopy. The results showed that extracts and compounds of the plant decreased percentage viability of cercariae in a dose-dependent manner.

Results: Within two hours of incubation, all cercariae died at the various concentrations of test compounds and extracts with the exception of methanol extract and the bioactive compound erythroivorensin at 15.6 μg/mL. The least potent extract, methanol, had an IC_{50} of 2.11±0.10 μg/mL. Eriodictyol, being the most active compound had an IC_{50} of 1.23 ± 0.05 μg/mL.

Conclusion: It is evident from the results obtained that fractions and isolated bioactive compounds of *Erythrophleum ivorense* can be a potential cercaricidal agent and therefore should be investigated further.

Keywords: Cercariae; schistosomiasis; erythroivorensin; eriodictyol; betulinic acid, Erythrophleum ivorense.

1. INTRODUCTION

Schistosomiasis also known as bilharziasis or snail fever is a parasitic disease caused by flukes (trematodes) of the genus Schistosoma. It is prevalent in tropical and subtropical areas, especially, in poor communities with no access to safe drinking water and adequate sanitation [1]. People become infected by being in contact with fresh water bodies infested with free-swimming larval forms of the parasite (cercariae) shed from freshwater snail intermediate hosts [2,3].

The disease is better known for its chronicity and debilitating morbidity which results in high costs in public health and economic productivity in developing countries [4]. Globally, more than 207 million people, 85% of whom live in Africa, are infected with schistosomiasis, and an estimated 700 million people are at risk of infection in 76 countries [5]. 200,000 deaths are globally attributed to schistosomiasis annually, and about 10 million women in Africa are infected during pregnancy [6].

There is no available vaccine currently and the chemotherapeutic agent of choice which is Praziquantel (PZQ), already faces drawback of drug resistance in some Schistosoma isolates [7, 8]. Complementing existing chemotherapy with synthetic molluscicides to eliminate the possibility of re-infestation of water bodies with cercariae faces the challenge of cost as well as environmental pollution [9]. It is based on these reasons that the search for affordable, readily available, less toxic schistosomicidal plant-derived products have become essential. This is because plants have timelessly served as good source for the discovery and development of newer drugs with about 25% of current medicines derived from them [10].

Artemisinin, quinine and licochalcone A are examples of plant-derived products in clinical use particularly against parasitic infections [11]. One of such promising plants is *Erythropleum ivolense* which is also known as 'potrodum' among the Akans in Ghana, and "Epoobo" among Yoruba people of South Western Nigeria. The stem-bark and roots of *E. ivorense* are particularly used in the treatment of convulsive pain, disorders, edema, emesis, constipation, smallpox as well as helminthic infestations [12]. A 70% ethanol extract of the stem bark of the plant has been reported to show moderate activity against a wide range of gram positive and gram negative organisms [13]. Wakeel et al., [14] reported on the anti-convulsant and sedative properties of *E. ivorense* stem bark extract. We have previously reported on the anti-inflammatory activity of the novel phytocompound, erythroivorensin, together with eriodictyol and betulinic acid isolated from the plant [15]. Additionally, we have earlier reported on the leishmanicidal activity of the root bark of the plant and identification of some of its compounds by ultra-performance liquid chromatography quadrupole time of flight mass

spectroscopy (UPLC-QTOF-MS/MS) [16]. Despite the fact that the effect of the leaf and stem bark extracts of *Erythrophleum ivorense* have been screened for antishistosomal activity against *Schistosoma mansoni* [17], this current research, in addition to using the various fractions of the root bark of the plant, focusses also on three isolated bioactive compounds: erythroivorensin, betulinic acid and eriodictyol against immature infective stage of *Schistosoma haematobium* Cercariae.

2. MATERIALS AND METHODS

2.1 Plant Collection and Extraction

The root bark of *Erythrophleum ivorense* was harvested from Adukrom in Nzema-East Metropolis of Ghana, in August 2017 and was authenticated using an earlier collected samples with voucher number BHM/Eryth/017R/2014, which had been deposited at the Herbarium unit of the Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, Kumasi-Ghana.

The root bark of *E. ivorense* collected was air dried at room temperature (25–27°C) for two weeks. The dried root bark was pulverized by milling into a coarse powder. 1 kg of the powdered air-dried root bark was cold macerated with 70% ethanol for 72 hours. The resulting extract was filtered and concentrated under reduced pressure (40ºC) using rotary evaporator (Buchi Rotavapor, R 200) to give a crude yield of 9% $^{w}/_{w}$. 80 g of the plant extract was successively partitioned with petroleum ether (4 L), ethyl acetate (4 L) and methanol (4 L) to obtain three fractions with the yield of 5.8 g, 22.7 g and 38.3 g respectively. Activity-guided isolation and characterization carried out as described previously [15] yielded the following pure compounds: erythroivorensin (**1**), betulinic acid (**2**) and eriodictyol (**3**) as shown in Fig. 1.

2.2 Collection of Snails

The snails, *Bulinus species*, the intermediate host for *S. haematobium*, were collected from endemic areas in their natural habitats from Tomefa along the Weija River in Ghana. The snails were kept in a plastic aquarium with 50 snails per each aquarium containing clean pond water at room temperature (25°C) and fed with lettuce at the Biomedical Science Laboratory of University of Cape Coast, Ghana. They were later washed with deionised water and examined for cercariae shedding using inverted microscopy as described previously by Amoani et al. [18].

2.3 *In vitro* Cercaricidal Activity Test

Cercaricidal effect of the various concentrations (15.6, 31.3, 62.5, 125.0, 250.0 and 500.0 µg/mL) of the crude (70% ethanolic) extract, its fractions (methanol, ethyl acetate and pet-ether) pure compounds (erythroivorensin, betulinic acid and eriodictyol) of the root bark of *E. ivorense* as well as control praziquantel were evaluated as described previously [4].

An average of 20 freshly shed cercariae were transferred into each of the 20 well plates (Costar) using micropipette. Various concentrations of the extracts and bioactive compounds were freshly prepared and transferred into one well on the plate. The negative control well contained the same number of cercariae and distilled water only. All experiments were carried out in triplicates. Mobility and viability of the Schistosoma infectious stage (cercariae) were observed for 3 hours.

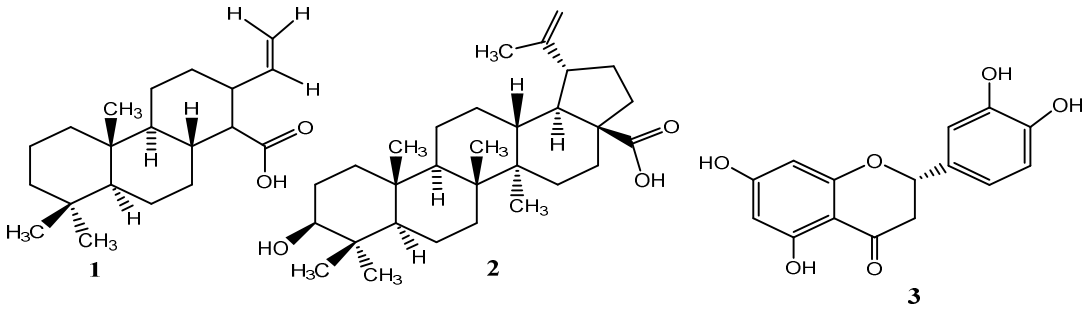

Fig.1. Chemical structure of erythroivorensin (1), betulinic acid (2) and eriodictyol (3) compounds isolated

Unaffected free-swimming larvae, immobile and dead cercariae at the bottom of the wells were observed at 4× magnification using an inverted microscope (Olympus CK 300). Survival and mortality at a successive interval of 15, 30, 60, 120, and 180 min were recorded. Cercariae were presumed dead when they stopped moving and sank down and their tail were detached.

The % viability was calculated using the equation below and this was used to plot the survival curves for each of the fractions and compounds.

$$\%\ Viability = \left(\frac{Initial\ count\ of\ live\ cercariae\ -number\ of\ dead\ cercaria}{Initial\ count\ of\ live\ cercariae}\right) \times 100$$

2.4 Statistical Analysis

Data was presented as mean ± standard error of mean (SEM). Graphpad® Prism Version 7.0 (Graphpad Software, San Diego, CA, USA) for Windows was used to perform all statistical analysis. Time-course curves of percentage viability of the plant extracts against time was plotted. The equation (1) above was used to calculate the percentage viability for each treatment. The concentration at which 50% of the cercariae were inhibited referred to as IC_{50} was determined by plotting a nonlinear regression curve (log concentration of inhibitor verses % viability).

3. RESULTS AND DISCUSSION

Exposure of *S. haematobium* cercariae to the crude hydro-ethanolic extract of *E. ivorense,* its fractions and compounds, showed concentration dependent increase in mortality (Figs. 2-4). The ethyl acetate fraction and one of its isolates, eriodictyol, showed higher mortality rate than the other fractions and compounds tested against the cercariae of *S. haematobium*. With the exception of erythroivorensin and betulinic acid (at 15.6 µg/mL), all the various fractions and eriodictyol at all concentrations achieved 100% mortality of cercaria within 180 min of incubation (Figs. 2 and 3). In the absence of the plant extract, cercariae showed normal viability without any morphological changes (tail loss) throughout the entire duration of the experiment as was observed in the control sample. Though 40% mortality of cercariae was achieved at the maximum concentration of praziquantel (PZQ 500 µg/mL), none of the various concentrations of the standard antischistosomal drug could eliminate all the cercariae.

The dose response curves of the effects of the various fractions and isolated compounds from *E. ivorense* on *S. Haematobium* cercariae demonstrates that the activity of these isolates and compounds are dose-dependent. The cercaricidal activities of the various fractions and extracts were quantified using $IC_{50.}$ From the results presented on Table 1 and Fig. 4, eriodictyol was found to be most potent with an IC_{50} of 1.23 µg/mL whereas the methanol fraction was found to be the least potent with IC_{50} of 2.11 µg/mL. The activity of the ethyl acetate fraction was higher than the total crude ethanol extract but lower than its isolate eriodictyol. Thus purification of the ethyl acetate fraction afforded higher anti-cercarial activity.

This current study investigated cercaricidal activities of methanol, alcoholic, petroleum ether, ethyl acetate fractions and isolated compounds (erythroivorensin, betulinic acid and eriodictyol) obtained from *Erythrophleum ivorense* on *Schistosoma haematobium* cercariae *in vitro*. We have earlier reported the anti-inflammatory and anti-leishmanial activity of these compounds and fractions from the plant [15, 16]. It is an indication that the plant will have an activity against cercaria from *Schistosoma haematobium*, another parasitic disease. Also, its anti-inflammatory property is essential since inflammation is an important component of infectious diseases [19]. The current study has demonstrated that the various fractions and compounds isolated from the plant have potent cercaricidal activity and that ethyl acetate fraction and the compound eriodictyol are the most potent. It is not surprising that the compounds cassane diterpene erythroivorensin, triterpene betulinic acid and flavanone eriodictyol which showed marked activity were all isolated from the ethyl acetate fraction of the plant.

The results obtained indicate a potent cercaricidal activity of the various fractions and compounds with ethyl acetate fraction and the compound eriodictyol being the most potent. The cassane diterpene erythroivorensin, triterpene betulinic acid and flavanone eriodictyol which showed marked activities were all isolated from the ethyl acetate fraction of the plant. The cercaricidal activity of the flavanone eriodictyol was relatively higher than that of the ethyl acetate fraction implying that the erythroivorensin and betulinic acid had a relatively little effect on the cercaricidal ability of the extract. The crude ethanolic extract comparatively recorded lower activity than its ethyl acetate fraction, probably

because some compounds, present in the root bark, may have antagonistically functioned to reduce the cercaricidal potency of the extract. That notwithstanding, the crude alcoholic extract, various fractions and isolated compounds produced 100% mortality of *Schistosoma haematobium* cercariae at higher concentrations within the 3 h study period.

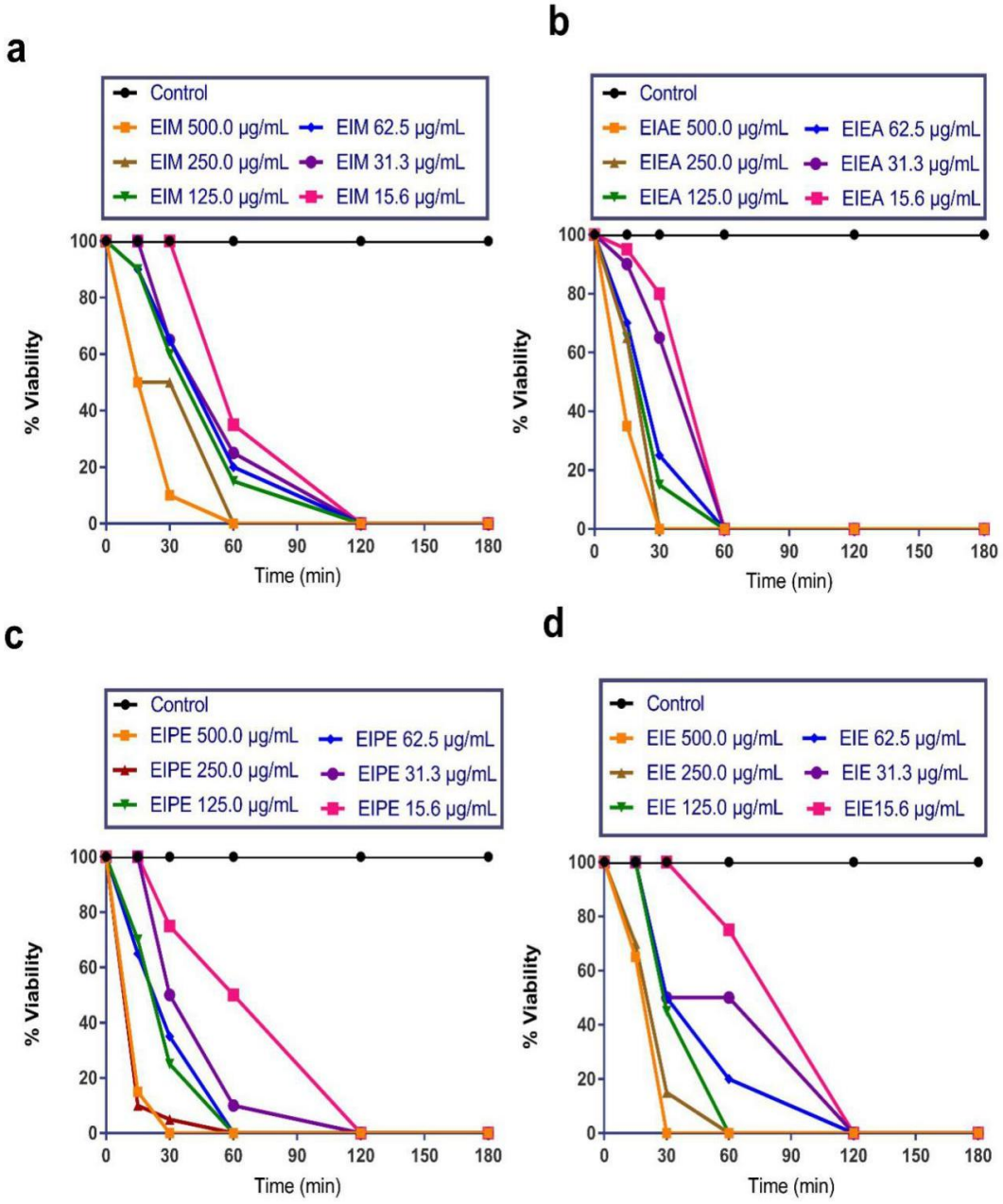

Fig. 2. Effect of different concentrations of (a) methanol (EIM) (b) ethyl acetate (EIEA) (c) petroleum ether (EIPE) fractions and (d) 70% crude ethanol (EIE) extract of *E. ivorense* root bark on the viability of *S. haematobium* cercariae

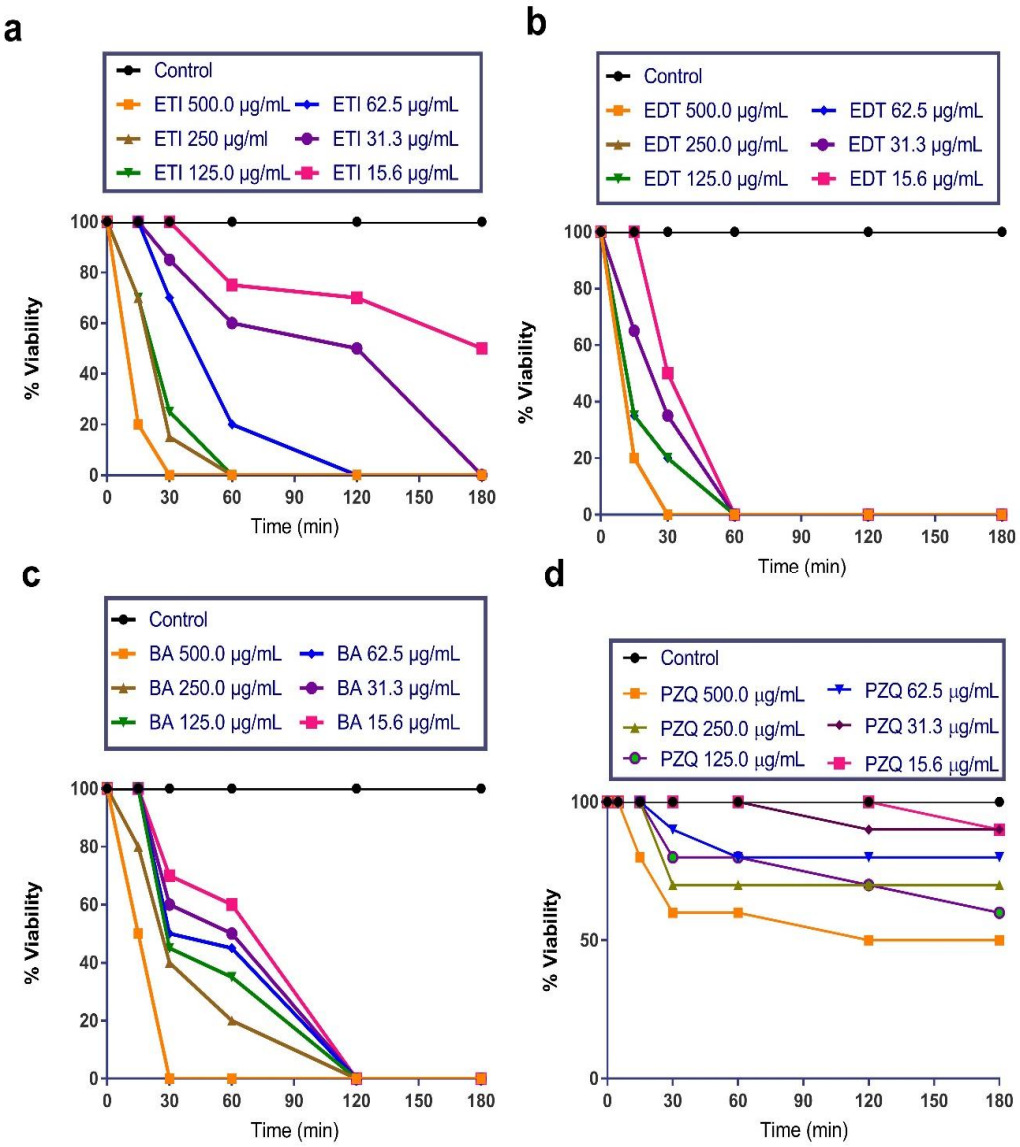

Fig. 3. Effect of different concentrations of (a) erythroivorensin (ETI) (b) eriodictyol (EDT) (c) betulinic acid (BA) isolated from the root bark of *E. ivorense* and (d) praziquantel (PZQ) on the viability of *S. haematobium* cercariae

Table 1. IC_{50} values of fractions and isolated compounds from *E. ivorense*

Compound/Fraction	IC_{50} (µg/mL)
EIE	1.75 ± 0.08
EIM	2.11 ± 0.10
EIEA	1.53 ± 0.02
EIPE	1.59 ± 0.03
ETI	1.92 ± 0.02
EDT	1.23 ± 0.05
BA	1.74 ± 0.10
PZQ	695.50 ± 0.05

Crude ethanol (EIE), Methanol (EIM), ethyl acetate (EIEA), petroleum ether (EIPE) extracts, erythroivorensin (ETI), eriodictyol (EDT), betulinic acid (BA) and Praziquantel (PZQ)

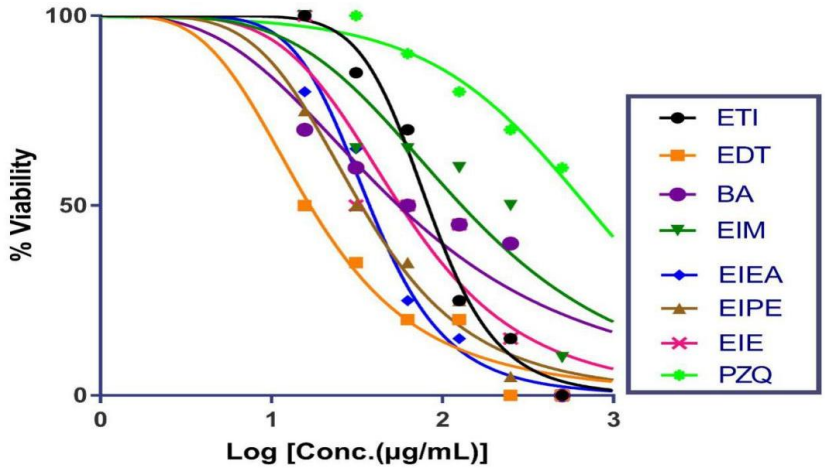

Fig. 4. Dose-response curves of the effects of the crude extract (EIE), various fractions (EIM, EIEA, EIPE) isolated compounds (ETI, EDT and BA) from *E. ivorense* and praziquantel (PZQ) on *S. haematobium* cercariae

Thus the present study has highlighted the ethyl acetate fraction and its flavanone constituent eriodictyol as clear drug candidates in the development of agents to obstruct the life cycle of the parasite through its asexual aquatic stage (cercaria) and thus could be considered in biological control programs. In Ghana and other African countries, due to the large dependence of the populace on herbal medicine use, consideration could be made in formulating the ethyl acetate fraction or eriodictyol as an ointment to be used prior to decent into these water bodies. Research into the safety of these products on other aquatic life is thus welcome.

Praziquantel, the most commonly used antischistosomal drug, increases the permeability of the membranes of Schistosome cells towards calcium ions. It induces contraction of the parasites which results in paralysis in the contracted state and also causes focal disintegrations [20]. However, this effect is not well expressed in cercariae hence its ineffectiveness against cercariae as was observed in the results presented in Fig. 3. The extracts and isolated compounds of *E. ivorense*, caused focal disintegration (loss of tail) and paralysis of the cercariae and subsequently, death. Further research on possible mechanism of action of the fractions and compounds isolated from the plant is recommended. Since standard anti-cercarial agents are not widespread, the present study brings to the fore, extracts, fractions and compounds of *E. ivorense* as potential biological drug leads for the development of eco-friendly cercaricides for the mitigation of schistosomiasis. This will help reduce the incidence and prevalence of the second most important human parasitic disease after malaria, on the wane.

4. CONCLUSIONS

The various fractions and compounds of *Erythrophleum ivorense* exhibited a marked cercaricidal activity. Thus, the study may provide some scientific justification for the ethnomedicinal uses of the root bark of *Erythrophleum ivorense* in Ghana. Therefore, it is recommend that the isolated bioactive compounds of this plant should be further evaluate and develop into a cercaricidal formulation for prophylactic use especially, before one descends into infested water body.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, Revised 1985) were followed. All protocols used in the study were approved by the Department of Biomedical Sciences' ethics committee.

DATA AVAILABILITY STATEMENT

The authors declare that all data have been included in the manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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