

***In Vitro* Cercaricidal Activity of Fractions and Isolated Bioactive Compounds from the Root Bark of *Erythrophleum ivorense* (Fabaceae) against *Schistosoma haematobium* Infection.**

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

Aim: This study investigated anticercarial 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* anticercarial 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 $\mu\text{g/mL}$) 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 erythroivorenin at 15.6 $\mu\text{g/mL}$. The least potent extract, methanol, had an IC_{50} of 2.11 ± 0.10 $\mu\text{g/mL}$. Eriodictyol, being the most active compound had an IC_{50} of 1.23 ± 0.05 $\mu\text{g/mL}$.

Keywords: cercariae, schistosomiasis, erythroivorenin, Eriodictyol, betulinic acid
Erythrophleum ivorense

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.

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-

37 swimming larval forms of the parasite (cercariae) shed from freshwater snail intermediate
38 hosts [2, 3].

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

45 There is no available vaccine currently and the chemotherapeutic agent of choice which is
46 Praziquantel (PZQ), already faces drawback of drug resistance in some *Schistosoma* isolates
47 [7, 8]. Complementing existing chemotherapy with synthetic molluscicides to eliminate the
48 possibility of re-infestation of water bodies with cercariae faces the challenge of cost as well
49 as environmental pollution [9]. It is based on these reasons that the search for affordable,
50 readily available, less toxic schistosomicidal plant-derived products have become essential.

51 This is because plants have timelessly served as good source for the discovery and
52 development of newer drugs with about 25% of current medicines derived from them [10].
53 Artemisinin, quinine, and **licochalcone A** are examples of plant-derived products which are in
54 clinical use particularly against parasitic infections [11]. One of such promising plants is
55 *Erythropheum ivolense* which is also known as ‘potrodum’ among the Akans in Ghana, and
56 “Epoobo” among Yoruba people of South Western Nigeria. The stem-bark and roots of *E.*
57 *ivorense* are particularly used in the treatment of convulsive pain, disorders, edema, emesis,
58 constipation, smallpox as well as helminthic infestations [12]. A 70% ethanol extract of the
59 stem bark of the plant has been reported to show moderate activity against a wide range of
60 gram positive and gram negative organisms [13]. Wakeel et al., [14] reported on the

61 anticonculsant and sedative properties of *E. ivorensis* stem bark extract. We have previously
62 reported on the anti-inflammatory activity of the novel phytochemical, erythroivorensin,
63 together with eriodictyol and betulinic acid isolated from the plant [15]. Additionally, we
64 have earlier reported on the leishmanicidal activity of the root bark of the plant and
65 identification of some of its compounds by ultra-performance liquid chromatography
66 quadrupole time of flight mass spectrometry [16]. Despite the fact that the effect of the leaf
67 and stem bark extracts of *Erythrophleum ivorensis* have been screened for antishistosomal
68 activity against *Schistosoma mansoni* [17], this current research, in addition to using the
69 various fractions of the root bark of the plant, focusses also on three isolated bioactive
70 compounds: erythroivorensin, betulinic acid and eriodictyol against immature infective stage
71 of *Schistosoma haematobium* Cercariae.

72 **2.0 Materials and Methods**

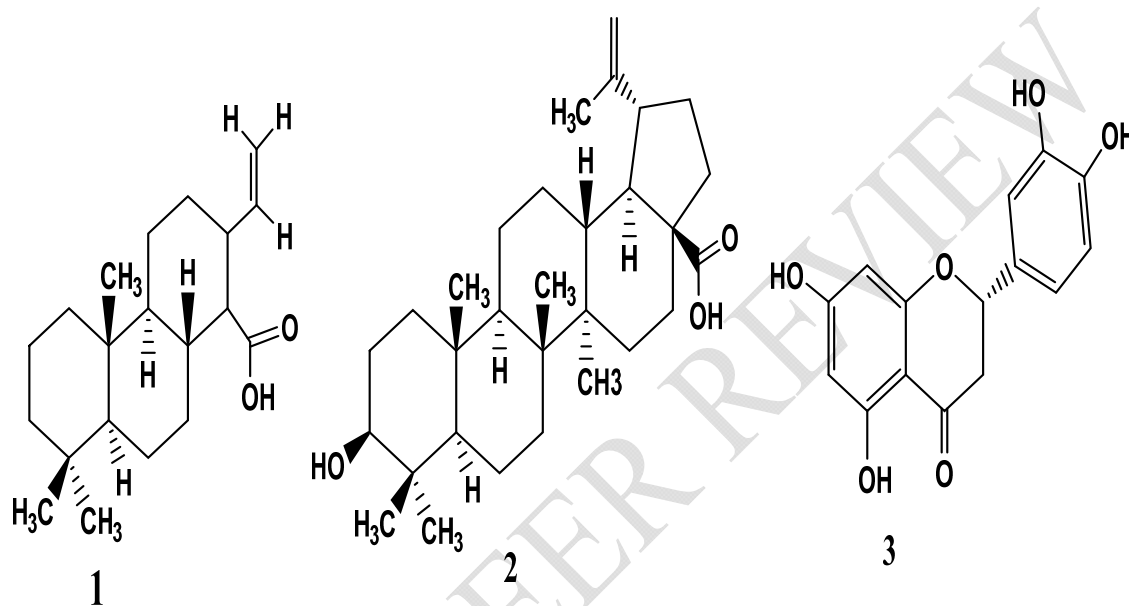
73 **2.1 Plant collection and extraction**

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

79 The root bark of *E. ivorensis* collected was air dried at room temperature (25–27 °C) for two
80 weeks. The dried root bark was pulverized by milling into a coarse powder. 1 kg of the
81 powdered air-dried root bark was cold macerated with 70% ethanol for 72 hours. The
82 resulting extract was filtered and concentrated under reduced pressure (40 °C) using rotary
83 evaporator (Buchi Rotavapor, R 200) to give a crude yield of 9% ^w/_w. 80 g of the plant extract

84 was successively partitioned with **pet ether** (4 L), ethyl acetate (4 L) and methanol (4 L) to
85 obtain three fractions with the yield of 5.8 g, 22.7 g and 38.3 g respectively.

86 Activity-guided isolation carried out as described previously [15] yielded the following pure
87 compounds: erythroivorensin (1), betulinic acid (2) and eriodictyol (3) as shown in Figure 1.



88

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

90

91 2.2 Collection of snails

92 The snails, *Bulinus species*, the intermediate host for *S. haematobium*, were collected from
93 endemic areas in their natural habitats from Tomefa along the Weija River in Ghana. The
94 snails were kept in a plastic aquarium with 50 snails per each aquarium containing clean pond
95 water at room temperature (25 °C) and fed with lettuce at the Biomedical Science Laboratory
96 of University of Cape Coast, Ghana. They were later washed with deionised water and

97 examined for cercariae shedding using inverted microscopy as described previously by
98 Amoani et al. [18].

99

100

101 **2.3 *In vitro* Cercaricidal Activity Test**

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

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

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

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

119
$$\% Viability = \left(\frac{\text{Initial count of live cercariae} - \text{number of dead cercariae}}{\text{Initial count of live cercariae}} \right) \times 100$$

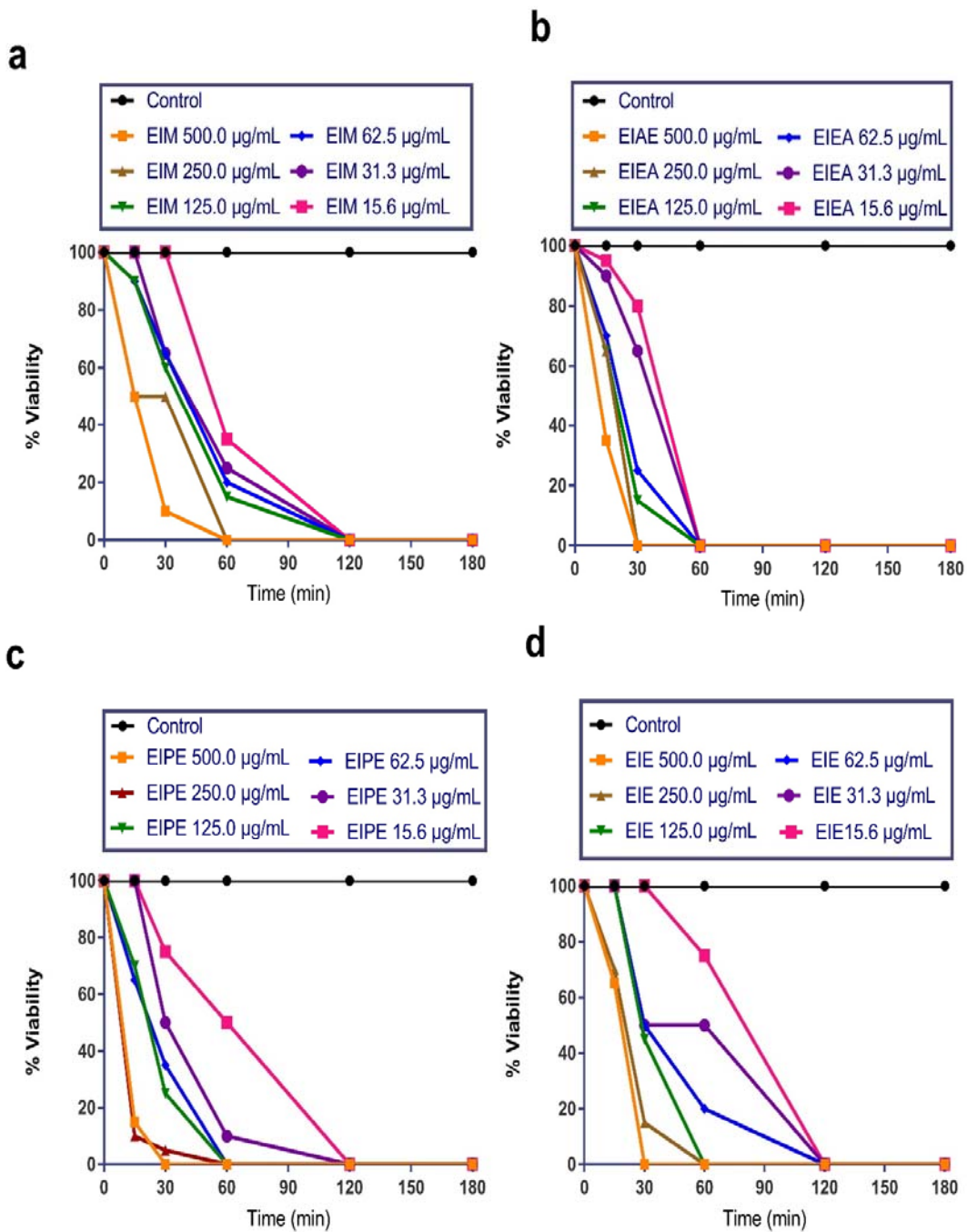
120 (1)

121 **Statistical analysis**

122 Data was presented as mean \pm standard error of mean (SEM). Graphpad® Prism Version 7.0
123 (Graphpad Software, San Diego, CA, USA) for Windows was used to perform all statistical
124 analysis. Time-course curves of percentage viability of the plant extracts against time was
125 plotted. The equation (1) above was used to calculate the percentage viability for each
126 treatment. The concentration at which 50% of the cercariae were inhibited referred to as IC₅₀
127 was determined by plotting a nonlinear regression curve (log concentration of inhibitor verses
128 % viability).

129 **3.0 Results and Discussion**

130 Exposure of *S. haematobium* cercariae to the crude hydro-ethanolic extract of *E. ivorensis*, its
131 fractions and compounds, showed concentration dependent increase in mortality (Figures 2-
132 4). The ethyl acetate fraction and one of its isolates, eriodictyol, showed higher mortality rate
133 than the other fractions and compounds tested against the cercariae of *S. haematobium*. With
134 the exception of erythroivorenin and betulinic acid (at 15.6 $\mu\text{g/mL}$), all the various fractions
135 and eriodictyol, at all concentrations, achieved 100% mortality of cercaria within 180 min of
136 incubation (Figures 2 and 3). In the absence of the plant extract, cercariae showed normal
137 viability without any morphological changes (tail loss) throughout the entire duration of the
138 experiment as was observed in the control sample. Though 40% mortality of cercariae was
139 achieved at the maximum concentration of praziquantel (PZQ 500 $\mu\text{g/mL}$), none of the
140 various concentrations of the standard antischistosomal drug could eliminate all the cercariae.

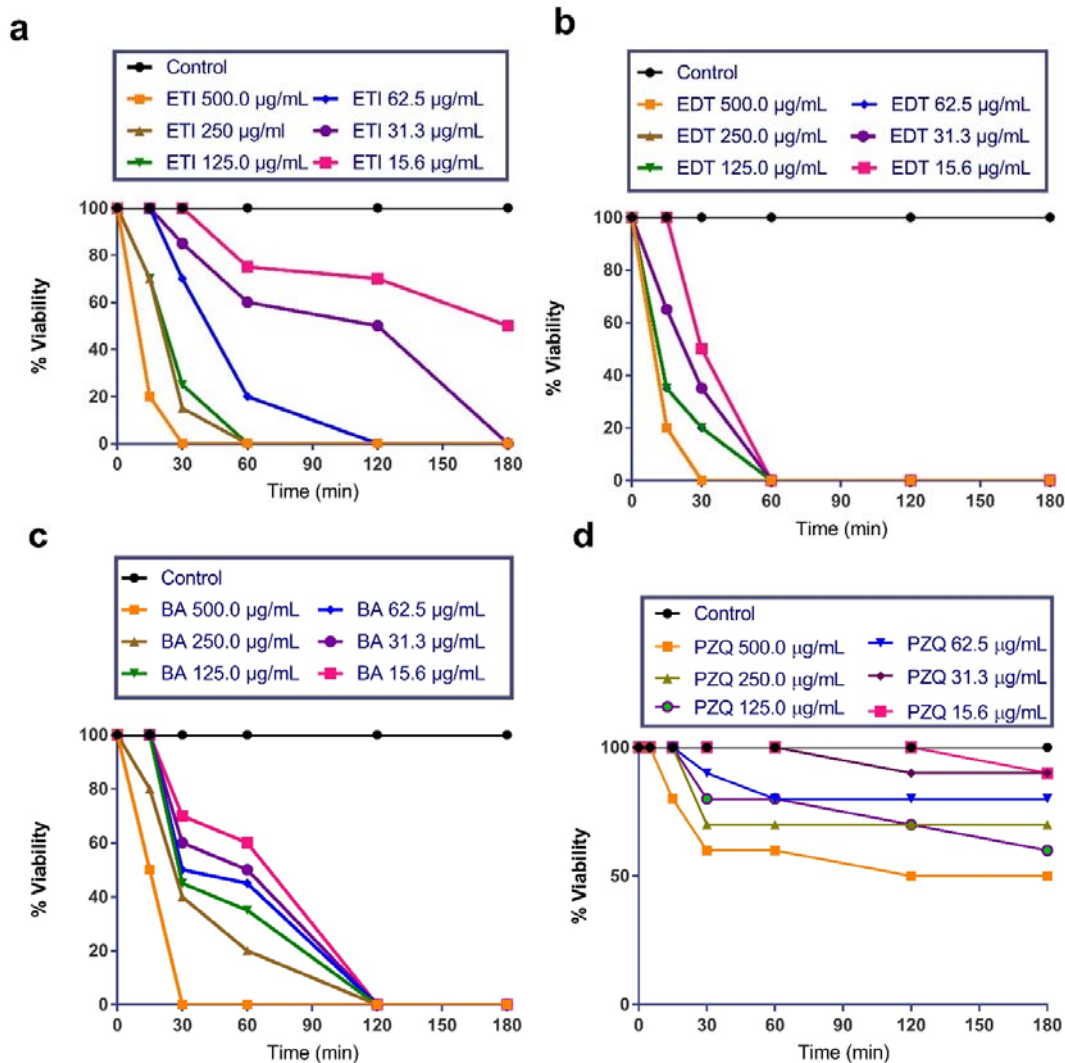


142

143 Figure 2: Effect of different concentrations of (a) methanol (EIM) (b) ethyl acetate (EIEA) (c) petroleum ether

144 (EIPE) fractions and (d) 70% crude ethanol (EIE) extract of *E. ivorensis* root bark on the viability of *S.*

145 *haematobium* cercariae



146

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

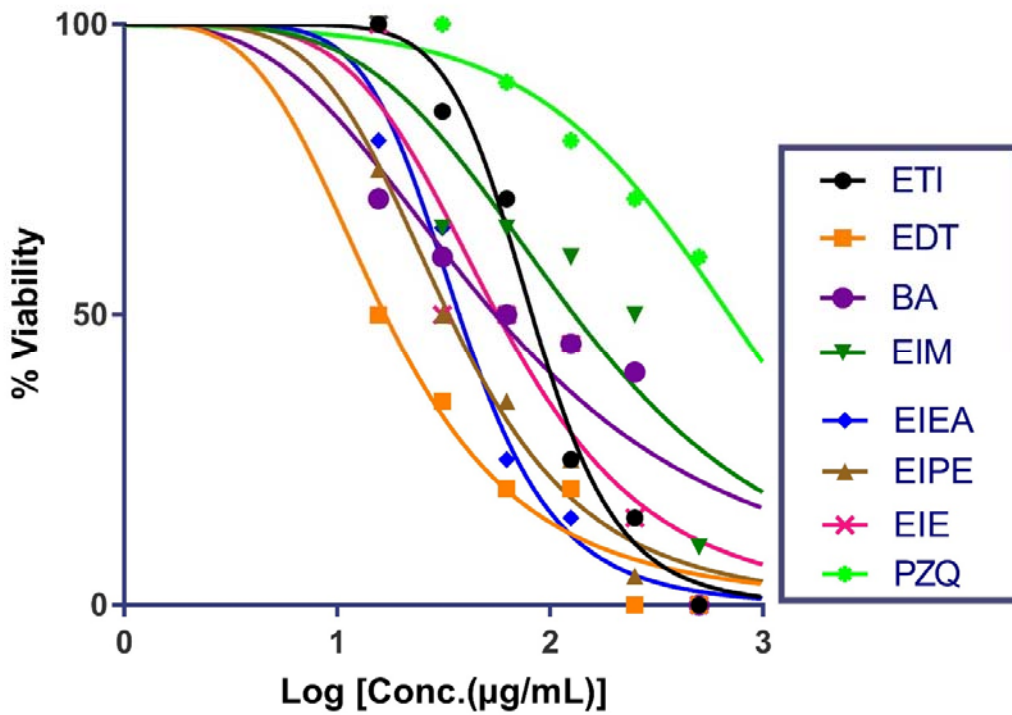
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151 The dose response curves of the effects of the various fractions and isolated compounds from
 152 *E. ivorense* on *S. haematobium* cercariae demonstrates that the activity of these isolates and
 153 compounds are dose-dependent. The cercaricidal activities of the various fractions and
 154 extracts were quantified using IC_{50} . From the results presented on Table 1 and Figure 4,

155 eriodictyol was found to be most potent with an IC_{50} of 1.23 $\mu\text{g}/\text{mL}$ whereas the methanol
156 fraction was found to be the least potent with IC_{50} of 2.11 $\mu\text{g}/\text{mL}$. The activity of the ethyl
157 acetate fraction was higher than the total crude ethanol extract but lower than its isolate
158 eriodictyol. Thus purification of the ethyl acetate fraction afforded higher anticercarial
159 activity.

160

161



162

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

165

166

167 Table 1. IC₅₀ values of fractions and isolated compounds from *E. ivorensis*.

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

168 Crude ethanol (EIE), Methanol (EIM), ethyl acetate (EIEA), petroleum ether (EIPE) extracts, erythroivorensin
 169 (ETI), eriodictyol (EDT), betulinic acid (BA) and Praziquantel (PZQ). This current study investigated
 170 cercaricidal activities of methanol, alcoholic, pet-ether, ethyl acetate fractions and isolated
 171 compounds; erythroivorensin, betulinic acid and eriodictyol obtained from *Erythrophleum*
 172 *ivorensis* on *Schistosoma haematobium* cercariae *in vitro*. We have earlier reported on the
 173 anti-inflammatory and anti-leishmanial activity of these compounds and fractions from the
 174 plant [15, 16]. It is an indication that the plant will have an activity against cercaria from
 175 *Schistosoma haematobium*, another parasitic disease. Also, its anti-inflammatory property is
 176 essential since inflammation is an important component of infectious diseases [19].

177

178 The current study has demonstrated that the various fractions and compounds isolated from
 179 the plant have potent cercaricidal activity and that ethyl acetate fraction and the compound
 180 eriodictyol are the most potent. It is not surprising that the compounds cassane diterpene

181 erythroivorensin, triterpene betulinic acid and flavanone eriodictyol which showed marked
182 activity were all isolated from the ethyl acetate fraction of the plant.

183

184 The results obtained indicate a potent cercaricidal activity of the various fractions and
185 compounds with ethyl acetate fraction and the compound eriodictyol being the most potent.

186 The cassane diterpene erythroivorensin, triterpene betulinic acid and flavanone eriodictyol
187 which showed marked activities were all isolated from the ethyl acetate fraction of the plant.

188 The cercaricidal activity of the flavanone eriodictyol was relatively higher than that of the
189 ethyl acetate fraction implying that the erythroivorensin and betulinic acid had a relatively
190 little effect on the cercaricidal ability of the extract. The crude ethanolic extract
191 comparatively recorded lower activity than its ethyl acetate fraction, probably because some
192 compounds, present in the root bark, may have antagonistically functioned to reduce the
193 cercaricidal potency of the extract. That notwithstanding, the crude alcoholic extract, various
194 fractions and isolated compounds produced 100% mortality of *Schistosoma haematobium*
195 cercariae at higher concentrations within the 3 h study period.

196

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

205

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

219 **4.0 Conclusions**

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

226 **Ethical consideration**

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

230 **Conflicts of Interest**

231 The authors declare that there is no conflict of interest regarding the publication of this paper.

232 **Data Availability Statement**

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

234

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