Immunomodulating potential of *Ekebergiacapensis* in murine *Schistosomamansoni* juvenile and adult infection

Abstract

Schistosomiasis is a neglected tropical disease occurring in sub-Saharan Africa and affecting almost 250 million people. The drug of choice for treatment for Schistosomiasis has been Praziquantel that has been used for many years and there is need to develop new drugs.The immunomodulatory potential of *Ekebergiacapensis* extract on both juvenile and adult *Schistosoma mansoni* infection in vivo was evaluated in this study.Swiss albino mice were infected individually with 90 *S. mansoni*cercariae and randomized into groups of five each for i) plant extract treated groups ii) positive control groups treated with conventional drugs PZQ or artemether iii) infected but untreated (negative control) groups. The mice were treated orally with aqueous extracts of *E. capensis* at doses of 200 and 400 mg/kg at 2 weeks (juvenile worms) and 7 weeks (adult worms) post infection. Immune enhancing potential of the medicinal plant was determined by analyzing the levels of cytokines in serum samples that were collected before and after treatment. A BD-Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 kit was used to quantitate the levels of cytokines using flow cytometer (FACS Calibur) and analysis of the data was done using FCAP software. Results indicated that the medicinal plant extract hadimmunomodulatory effect. There was a significant increase ($P<0.05$) in Th1 cytokines (IL-2, IFN- γ and TNF-α), a decrease in Th2 cytokines (IL-4, IL-6 and IL-10) and an increase in Th17 (IL-17). These findings confirm the potential use of medicinal plants in the management of schistosomiasis.

Key words: Schistosomiasis, Praziquantel, Schistosoma mansoni, Artemether, Ekebergiacapensis Immunomodulatory, Cytokines

Comment [OO1]: italics

Comment [OO2]: italics

Introduction

Schistosomiasis is one of the neglected tropical diseases caused by helminthic flatwormsof the genus *Schistosoma* that reside in the blood vasculature and produce eggs that result in pathology. It is endemic in 78 countries, with over 90% of cases occurring in sub-Saharan Africa and an estimated 250 million people require preventative treatment (WHO, 2023). The disease remains second to malaria in terms of socio-economic impact in tropical and subtropical areas (Carter, 2008).

Schistosomiasis is contracted during normal activities like swimming, bathing, fishing, and farming where schistosome cercariae released by infected intermediate (snail) hosts penetrate the skin of the mammalian host transforming into a schistosomula. The schistosomula migrates to the lungs and penetrates the pulmonary capillaries to be carried to the systemic circulation and to the portal system. In the hepatic circulation, they mature into adults, pair up and migrate to the mesenteric veins where they mate and eggs are excreted in feces for intestinal schistosomiasis. The eggs hatch in water into miracidia which penetrate the intermediate snail hosts for the cycle to continue (CDC, 2010).

The immune response during acute *S. mansoni* infection (caused by schistosomula and juvenile worms) at around 4-5 weeks is T helper type 1 (Th1) with the expression of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α)interferon-gamma (IFN-γ), and the interleukins (IL) IL-1, IL-2, and IL-12and a dry cough, fever, angioedema and blood eosinophilia is experienced during this stage (Jauréguiberry et al., 2010). T helper 2 (Th2) is induced by soluble egg antigens (SEA) from 5-6 weeks with the onset of egg deposition causing a shift from Th1 to Th2 characterized by IL-4, IL-5, IL-10, IL-13 and immunoglobulin E (de Jesus et al., 2002; Fallon et al., 1998; Stadecker& Hernandez, 1998; Fallon, 2000). The Th2 response peaks at 8-10 weeks and is responsible for the coordination of granulomatous inflammation (Fallon et al., 2000; Brunet et al.,1997; Phythian-Adams et al., 2010) and also dampens the Th 1 component (Grzych et al., 1991). The Th17 on the other hand is at low levels compared to Th1 and Th2 responses and emerges in CBA mice and not BALB/C or C57BL/6 (Kalantari et al., 2019) and has been reported to promote immune pathology rather than benefit the host (Rutitzky et al., 2009). As the disease progresses there is a immunoregulation of the Th1/Th2 balance by the T regulatory cells (Tregs) and B regulatory (Bregs) that causes a reduction of Th2 inflammation via an IL-10-mediated pathway (van der Vlugt et al., 2014).

Praziquantel has been the main drug of choice for the treatment of all species of schistosomes because of its efficacy, ease of administration, safety, and cost (Cioli et al., 2003). There are however concerns about decreased efficacy and the emergence of resistance with continued use (Kabuyaya et al., 2018). It is effective against adult worms but not juvenile worms which go on to establish infection together with the associated morbidity. The World Health Organization's (WHO) neglected tropical diseases (NTDs) roadmap targets to eliminate schistosomiasis by 2030 (WHO, 2021) and there is a need for the development of novel natural therapeutics against schistosomiasis (Caffrey,2007) that are cheap and readily available, effective on all stages of schistosomes or that can be used in combination with PZQ. Such organically derived drugs may act as antimicrobial agents or indirectly boost cellular and humoral immunity against the organism (Amer, 2006).

Comment [OO3]: Reference this please

Medicinal plants have been in use for a long time because they are a valuable source of pharmacological properties, are inexpensive and are available to people who do not have access to conventional drugs. The plant *Ekebergiacapensis*Sparm from the family Meliaceae (The Mahogany family) has been used traditionally to treat many ailments and it is widely distributed in the Central and Nyanza regions of Kenya (Gachathi, 2007),~~and~~ Uganda, Ethiopia, Zimbabwe, Swaziland and South Africa. The plant has been studied and reported widely for its medicinal properties. The in vivo antischistosomal potential of both *E. capensis*and *Azadiractaindica* (neem) against juvenile and adult infections was evaluated in a study in which *E. capensis*showed more potency in reduction of both the worm burden and tissue egg load at both stages (Musili et al., 2015). This study therefore determined theimmunomodulatory potential of *E. capensis* in Swiss albino mice infected with*S. mansoni* at juvenile and adult stages. The immunomodulating effect of these herbs was assessed by measuring levels of circulating cytokines before and after treatment with the herbal extracts.

Comment [OO4]: Meaning?

Material and Methods

This experimental study was carried out in the Animal house facility, Schistosomiasis and Immunology laboratories at the Kenya Medical Research Institute.

Maintenance of *S. mansoni*parasite

S. mansoni cercariae were obtained from 3 Swiss albino mice which were already infected and on life cycle maintenance at the Institute. Livers from 3 mice were emulsified and filtered to obtain a filtrate which was then illuminated using a lamp. After hatching into miracidia, 30 *Biomphalariapfeifferi* snails of 4mm diameter were infected using a routine optimized technique. The infected snails were maintained in freshly prepared aquariums and maintained with lettuce and supplemented with bone meal for 28 days.

Infection of mice

Infected snails were collected from the aquaria and placed in 50ml beakers filled to 1/3 with dechlorinated water and exposed to artificial light to enhance cercariae shedding for 2 hours. To avoid unisexual infection, more than 25 snails were used. To enumerate cercariae produced, 5, 50µl subsamples of the cercariae suspension were obtained after gently swirling the beaker and were stained with Lugol's iodine on a Petridish. 60 male Swiss albino mice aged 6-8 weeks old and weighing 20-22g were infected with 90 cercariae each using the abdominal ring method (Smithers and Terry, 1965). The mice were maintained with mice pellets and water ad libitum.

Study groups

The infected mice were randomized into groups of 5 mice each and placed in separate cages representing the juvenilestage at 2 weeks post infection (pi) and adult stage at 7 weeks pi using two different concentrations of the medicinal plant extract (200mg/kg and 400mg/kg). The control groups were included in the experiment and were the infected untreated group (negative control) and two positive control groups of juvenile infection (200mg/kg of artemether) and adult infection (200mg/kg of PZQ).

Extraction of plant and treatment

The back of the *E. capensis* was collected from the Mount Kenya Forest and was taken to the East African Herbarium in Nairobi for cataloging and voucher specimens deposition (*E. capensis*: Stem bark (Ec-SB/04) 26). The water extraction of plant bark was done at the Institute's Centre for Traditional Medicine and Drug Research.Treatment was done by administering the mice with two doses (200mg/kg and 400mg/kg) of the herbal extracts orally by gastric gavage using stainless steel needles. Two positive control groups were treated with artemether at 200mg/kg administered orally by gastric gavage once a day for 3 consecutive days using an oral volume of 0.2 ml per mouse (Utzinger et al., 2002) for comparison with the juvenile group and another one with PZQ at a dose of 200 mg/kg body weight per day using a dose volume of 0.05 ml for 5 consecutive days to a total dosage of 1000 mg/kg (Gönnert, 1977) for comparison with the adult group since PZQ targets adult worms.

Collection of blood

On the day of treatment, blood samples were collected from the tail ends of all the infected mice on days 14 and 49 post-infection (pi) from juvenile and adult worm groups respectively then treatment was started on the same day (a few hours after bleeding). Blood samples were collected again one day before perfusion that is on days 41 and 69 from juvenile and adult worm groups respectively. Briefly, the tip of the tail was nicked off (2-3mm) using a sterile sharp pair of scissors. The tail was gently massaged and blood was collected drop by drop into a sterile 300µl Eppendorf tubes. This procedure was performed ascetically to avoid infection. To enhance blood flow, the mice were placed near a table lamp or their tails were dipped in warm water to

dilate the tail veins. All blood samples were placed on ice to enhance blood clotting and spun in a microfuge at 1500rpm for 30 minutes to separate serum. The serum was pipetted out using a micropipette and sterile tips into 200µl storage tubes, labeled, and stored at -80^{0}C until analysis of cytokine profiles. A maximum volume of 300µl was collected from each mouse during each bleed. For collection of the second blood sample (before perfusion), the wounds at the tips of the tails were reopened by cutting 2-3mm using a sterile pair of scissors and bled the same way as before.

Cytometric Bead Array (CBA) assay

Cytokine analysis was done using the BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit which allowed for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A. Aliquots of sera that had been frozen at -80^{0}C were thawed and CBA analysis performed as per the manufacturer's instructions.

The samples were acquired on the flow cytometer the same day they were prepared since prolonged storage of the samples once the assay is complete would result to increased background and reduced sensitivity. To facilitate analysis of samples using FCAP Array software, the manufacturer's recommendations were followed in that the samples were acquired from the lowest (0 pg/ml) to the highest (Top Standard) concentration followed by the test samples. All the Flow Cytometer Standard (FCS) files (standards and samples) were stored in a single folder for analysis using FCAP v3 software.

Statistical Analysis

The Mouse Th1/Th2/Th17 Cytokine data was analyzed using FCAP v3 Array Software and the results were saved in Excel sheets for statistical analysis to be conducted. The mean level of cytokines in the groups was also subjected to Student's t-test using Microsoft Excel® to determine their statistical significance in comparison with the control groups. The data was considered significant if $P<0.05$, highly significant if $P<0.01$ and very highly significant if $P<0.001$.

RESULTS

Effect of treatment with *E. capensis* at 7 weeks p.i on Th1/Th2/Th17 cytokines

We examined the expression of Th1/Th2/Th17 cytokines which are responsible for immune reaction during acute *S. mansoni* infection inorder to determine the immunomodulatory effect of *E. capensis* extract at the two different stages of the disease.The CBA assay showed that *E.capensis* at a concentration of 400mg/kg and 200mg/kg body weight at adult (7weeks) infection resulted in a varied effect on the levels of the Th1/Th2/Th17 cytokine when compared to the infected untreated group as shown in Table 1. At 400mg/kg, *E. capensis* caused an increase in the mean serum levels of IFN-γ (124.7pg/ml, $P<0.001$), IL-2 (14.1pg/ml, $P<0.5$) at and Th17(113.8pg/ml, $P<0.01$) whereas, a decrease in TNF-α (27.4pg/ml, $P<0.001$), IL-4 (60.8pg/ml, $P<0.01$), IL-6 (20.5pg/ml, $P<0.01$) and IL-10 (53.8pg/ml, $P<0.001$) was noticed.

The extract at 200mg/kg showed a reduction in TNF-α, IL-4, IL-6 and IL-10 at 31.6pg/ml ($P<0.001$), 41.3pg/ml ($P<0.001$), 18.5pg/ml ($P<0.01$) and 72.8pg/ml ($P<0.001$) respectively when compared to the infected untreated group and an increase in IFN-γ at 111.5pg/ml

(P<0.001), IL-2 at 14.04pg/ml (P<0.05) and a dramatic increase in the level of IL-17 at 131.9pg/ml P<0.001). This was comparable to the PZQ treated group as shown in Table 1.

Table 1: Mean serum levels of cytokines following treatment with *A. indica* and *E. capensis* 7 weeks p.i.

	Th1			Th2			Th17
	TNF	**1FN**	**IL-2**	**IL-4**	**IL-6**	**IL-10**	**IL-17**
Infected untreated (Control)	41.6±1.0	65.8±0.9	5.8±0.3	86.1±1.8	28.01±1.7	93.1±2.6	55.9±1.2
PZQ, 200mg/kg	15.5±0.8 (□□□, ***)	118.0±1.9 (□□□,***)	21.1±1.4 (□□, **)	56.2±1.1 (□□□, ***)	18.5±0.9 (□□□, **)	44.8±1.3 (□□□, ***)	108.6±3.6 (□□□,**)
EC,400mg/kg	27.4±0.9 (◊◊◊, ***)	124.7±3.1 (□ □, ◊, * * *)	14.1±2.0 (□, ◊◊, *)	60.8±2.2 (□□,◊, ***)	20.5±0.6 (□, ◊, **)	53.8±1.3 (□□□,◊◊◊,***)	113.8±4.8 (□,◊,**)
EC,200mg/kg	31.6±0.9 (□□□,◊◊◊,***)	111.5±2.1 (□□□,◊,***)	14.04±2.2 (□,◊,*)	41.3±1.4 (□□□,◊◊◊,***)	18.5±1 (**)	72.8±1.4 (□□,◊◊◊,***)	131.9±1.2 (□□□,◊◊,***)

Data are expressed as mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript: □comparison between before treatment and after treatment◊comparison with PZQ,*comparison with infected untreated (control) group. □ or ◊ or *significant (P<0.05), □□ or ◊◊ or **highly significant (P<0.01) and □□□ or ◊◊◊ or ***very highly significant (P<0.001)

Effects of treatment with*E. capensis* on cytokine profiles (2 weeks pi)

Treatment was also done using the plant extract to target juvenile worms and using artemether as a positive control drug for comparison. At 2 weeks post infection, there was a general increase in Th1 cytokines, a reduction in Th2 cytokines and a moderate increase in Th17 cytokine when the mice were treated with *E. capensis* at both concentrations of 400mg/kg and 200mg/kg when compared to the infected untreated control group as shown in Table 2. Again, the effect of the extract at both concentrations on the cytokines gave a pattern that was comparable to the artemether treated group. There was an increase in Th1 cytokines after treatment with *E. capensis* at 400mg/kg whereby TNF-α was recorded at a level of 86.7pg/ml (P<0.001), IFN-γ at 106.9pg/ml (P<0.5), IL-2 at 15.9pg/ml (P<0.001), and an increase in the level of IL-17 to 62.4pg/ml (P<0.01). The Th2 cytokines reduced to 30.6pg/ml (P<0.001), 6.1pg/ml and 50.3pg/ml (P<0.001) for IL-4, IL-6 and IL-10 cytokines respectively when compared to the infected untreated group.

At 200mg/kg body the level of IL-17 increased to 83.9pg/ml while the Th1 cytokines also increased to 90.6pg/ml for TNF- α, 119.7pg/ml for IFN- γ and 17.8pg/ml for IL-2. On the other hand, the Th2 cytokines reduced to 24.7pg/ml, 5.1pg/ml and 50.3pg/ml for IL-4, IL-6 and IL-10 respectively when compared to the infected untreated control group.

Table 2. Mean serum levels of cytokines following treatment with *A. indica* and *E. capensis* 2 weeks p.i.

Th1	Th2	Th17

Comment [OO5]: Was this mentioned in your assay methodology?

	TNF	1FN	IL-2	IL-4	IL-6	IL-10	IL-17
Infected untreated (Control)	54.7±0.7	77.9±1.4	1.02±0.1	85.7±1.6	8.9±0.2	67.4±1.5	55.9±1.9
ART,200mg/kg	87.5±1.1 (¤¤¤,***)	142.5±2.5 (¤¤¤,***)	16.9±0.3 (¤¤¤,***)	44.2±0.8 (¤¤¤,***)	6.2±0.4 (¤¤,**)	54.7±0.4 (¤¤¤,**)	59.4±1.0 (¤¤¤)
E.C,400mg/kg	86.7±0.9 (¤¤¤, ***)	106.9±5.6 (¤, ◊◊, *)	15.9±0.1 (¤¤¤,◊,***)	30.6±0.7 (¤¤¤,◊◊◊,***)	6.1±0.2 (***)	50.3±0.8 (¤¤, ◊◊, ***)	62.4±0.9 (¤¤¤, ◊, **)
E.C,200mg/kg	90.6±0.8 (¤¤¤, ◊, ***)	119.7±0.9 (¤¤¤,◊◊◊, ***)	17.8±0.5 (¤, ***)	24.7±1.6 (¤¤¤,◊◊◊,***)	5.08±1.8 (¤¤¤,◊,***)	50.3±1.0 (¤¤¤,◊,***)	83.9±0.5 (¤¤¤,◊◊◊,**)

Data are expressed as mean±SD, n=5. Data were analyzed by t-test: two sample assuming unequal variance. Values with superscript: ¤comparison between before treatment and after treatment◊comparison with ART (Artemisinin),*comparison with infected untreated (control) group. ¤ or ◊ or *significant (P<0.05), ¤¤ or ◊◊ or **highly significant (P<0.01) and ¤¤¤ or ◊◊◊ or ***very highly significant (P<0.001).

DISCUSSION

While schistosomiasis remains a major public health problem of great socioeconomic impact (Hajissa et al., 2018), PZQ remains the only available drug for the treatment of all human schistosomiasis species (Cioli, 2003). Antimonials were in use in the 1918 but had numerous side effects (Cioli et al., 1995). This resulted in the search for metal-free drugs as alternatives (Katz & Coelho, 2008). In the 1960s there was a breakthrough in the treatment of schistosomiasis with the advent of metrifonate, nitrofurans, lucanthone, niridazole, hyacanthone and finally oxamniquine. In 1970s, several antischistosomal drugs emerged such as tubercidin, amoscanate, PZQ and its benzodiazepine derivative Ro11-3128 and oltipraz. The therapeutic doses of most of these drugs were found to cause major side effects (Cioli et al., 1995; Fenwich& Webster, 2006).

Since PZQ has been used to treat individuals and in mass drug administration (MDA) programs (Lago et al., 2018) for a long period and there are fears of emergence of reduced efficacy or emergence of resistance with continued use (Kabuyaya et al., 2018). There have been reports of reduced efficacy in response to MDA (Wang et al, 2012) although this might also be caused by high infection intensities, low drug absorption, high rates of reinfections and minimal immunological response to previous infections (Danso-Appiah & De Vlas, 2002). There are no documented alternative treatments to PZQ and there is a need to develop other drugs for schistosomiasis and medicinal plants are a preferred alternative.

This study aimed at determining the immunomodulating effect of aqueous extracts of*E. capensis* which had shown appreciable antischistosomal activity in both adult and juvenile infections in mice in vivo (Musili et al., 2015). Effect of treatment with the *E. capensis* on cytokine profiles was analyzed for the two doses (200mg/kg and 400mg/kg). The analysis was done using BD-CBA mouse Th1/Th2/Th17 cytokine kit.

Comment [OO6]: Kindly look for documented cases of resistance to PZQ and reference.

Comment [OO7]: There is no need for this here.

At 7 weeks p.i, treatment with *E. capensis* at 200mg/kg and 400mg/kg resulted in a reduction in TNF-α but an increase in IFN-γ and IL-2 (Th1 cytokines). Effect on Th2 cytokines resulted in a reduction in IL-4, an increase in IL-6 and a reduction in IL-10 levels. There was however an increase in IL-17 at both doses, and this was comparable to the observation in the PZQ treatment group. The effect of the plant extract on juvenile *S. mansoni* infection (2 weeks p.i) resulted in an increase in TNF-α, IFN-γ and IL-2 cytokines. There was a reduction in Th2 cytokines IL-4, IL-6 and IL-10 and an increase in IL-17 (Th17) cytokine. At 2 weeks p.i, a Th1 immune response was maintained due to death of larval worms after treatment and therefore fewer eggs being laid by the surviving worms. An interesting observation was that Th1 and Th17 cytokines increased in most of the treatment groups while Th2 cytokines decreased. A decrease in IL-10 also seemed to result in an increase in IL-17.

Both plants were found to modulate the course of schistosome infection at 7 weeks and 2 weeks p.i with an interesting trend that showed an increase in Th1, a decrease in Th2 and an increase in Th17 cytokines. The predominance of Th1 cytokines that was observed in this study could be as a result of helminthotoxic effect of these medicinal plant extracts to schistosomulae thus preventing/ reducing the development of egg laying adult worm pairs (2 weeks p.i). A reduction in worm burden could have resulted in down regulation of egg induced Th2 response and maintenance of Th1 predominant cytokine profile characterized by high IFN-γ and low IL-4. IFN-γ has been shown to be involved in protective immunity to schistosomiasis in murine models (Hewitson et al., 2005). Maintenance of high levels of Th1 and Th17 cytokines and low levels of Th2 cytokines is also indicative of a failure of a switch from Th1 to Th2 due to the death of worms after treatment and therefore fewer eggs being laid by the surviving worms.

Comment [OO8]: What are the 2 plants you used in this study or was this from a previous study?

Murine schistosomiasis is characterized by Th1 reaction (with a predominant secretion of IFN-γ, minimal level of IL-4 and IL-5) occurring during prepatency and then shifting to a Th2-based profile which develops after the onset of oviposition and persists throughout the acute phase of infection (with high IL-4 and IL-5, but low IFN-γ) (Davies et al, 2004). Ironically, egg induced Th2 responses are an immunologic double-edged sword, participating in protection of host tissues from egg-induced injury (Brunet et al.,1997) and in the development of the egg-induced pathology and fibrosis associated with chronic schistosome infection (Wynn and MacDonald, 2004). The natural and induced forms of severe schistosomiasis correlates with high levels of pro-inflammatory cytokines IFN-γ and IL-17 (Rutitzky et al., 2008). This is indicative of the Th1 and Th17 subpopulations of CD4 T lymphocytes. This can be related to the observation in this study. The shift from Th2 to Th1-like immune response (as observed in this study) is essential for the down modulation of granuloma reaction and disease control. Th1 cytokine profile results in the development of smaller granulomas (Brunet et al, 1998). PZQ activity has been shown to be dependent on T cell mediated immunity (Ammann et al., 2004). IFN-γ is involved in protective immunity to schistosomiasis in murine models. The results from this study are supported by studies that have been carried out on *A. indica* showing that it has immunomodulation ability. The aqueous extracts of neem leaves have been shown to have immunomodulatory response to live Newcastle disease vaccine (Garbaa et al, 2013). Neem leaf preparation enhances Th1 immune response and anti-tumour immunity against breast tumour associated antigen (Mandal-Ghosh et al, 2007). There is no evidence of previous studies on the immunomodulatory effect of *E. capensis*, thus this is the first one.

Comment [OO9]: Reference

The results from this study ~~show~~showed that *E. capensis*hasan appreciable ~~immune enhancing~~immune-enhancing ability. Further studies should be done to determine if the extracts from this plant can be used singly or in combination with PZQ in the management of schistosomiasis since this could lead to effective and targeted therapies and also become a strategy for transmission control which can reduce the morbidity of schistosomiasis in endemic regions. Isolation and characterization of the active compound(s) of these plants and determination of their mechanism(s) of action is also recommended in search for novel antischistosomal agents.

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