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Evaluation of Cercaricidal and Miracicidal Activity of Selected Plant Extracts Against Larval Stages of Schistosoma Mansoni

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ABSTRACT
Schistosomiasis is a parasitic disease caused by blood-flukes of the genus *Schistosoma.* It is one of the most widespread of all human parasitic diseases, ranking second only to malaria in terms of its socioeconomic and public health importance in tropical and subtropical areas.More than 207 million people, 85% of whom live in Africa, are infected with schistosomiasis,and an estimated700 million people are at risk of infection in 76 countries.Control of schistosomiasis faces serious drawbacks of emergence of drug resistant parasites and molluscicide resistant snail hosts.Due to improper waste disposal,infected faecal matter enter water bodies such as canals rivers and springs where miracidia that hatch from parasite eggs develop into cercariae inside snail intermediate hosts and are infective to humans upon release in to the water.This study sought to evaluate the miracicidal and cercaricidal activity of selected plant extracts on larval stages of *Schistosoma mansoni*.Ten cercariae and miracidia were exposed to extract concentrations ranging from 10-150ppm.The most active extracts were *Phytolacca dodecandra* (LT_{50} 10.84 and16.91minutes) and *Solanum linaeanum* (LT_{50} of 22.86 and 26.96 minutes) respectively that killed 50% of miracidia and cercariae in less than 30minutes.This was followed closely by *Solanum americanum* (LT_{50} 31.02 and 31.89) and *Anonna squamosa* LT_{50} 35.29 and 40.46minutes respectively.*Piper nigrum* was the least active recording LT_{50} 46.84 and 56.75 of miracidia and cercaria respectively.Miracidia were more susceptible to extracts than cercariae.The higher susceptibility of miracidia to extracts has also been reported in other studies and it is advantageous since killing one miracidium prevents the formation of thousands of cercariae which are infective to humans.All the extracts killed larvae within one hour at concentration less than 100ppm and could be categorized as potent cercaricide and miracicides.
Keywords: Miracidal, Cercaricidal,Schistosomiasis,Phytolacca *dodecandra,Solanum linaeanum,Solanum americanum,Anonna squamosa* and *Piper nigrum*

5.1 INTRODUCTION

Schistosomiasis or snail fever is an acute and chronic parasitic disease caused by blood flukes (trematode worms) of the genus *Schistosoma.* It is the second most devastating tropical disease in the world, in terms of morbidity and mortality. It is prevalent in tropical and subtropical areas, especially in poor communities without access to safe drinking water and adequate sanitation (WHO, 2016). People get infected by being in contact with fresh water bodies that harbor free-swimming larval forms of the parasite (cercariae) shed from freshwater snail intermediate hosts (Naples *et al.,*2005 and Mengistu *et al.,*2011). In Kenya the disease is transmitted mainly by *Biomphalaria pfeifferi* to people doing agricultural work in irrigation farms, domestic chores, and recreational activities which expose them to water harboring infected snails. Globally, about 207 million people are infected with Schistosomes and about 600 million are at risk (WHO, 2015; Ojewole, 2004).

Schistosomiasis transmission can be interrupted at four distinct points: Preventing human feacal matter from reaching fresh water (sanitation),preventing the interaction of free swimming larval stages of schistosomes (miracidia, cercariae) with intermediate host snail (broadly-snail control), preventing exposure to susceptible humans to the infective, free swimming cercariae (reduction of water contact),chemotherapeutic attack on the parasite population living within human host (mass or targeted chemotherapy).

At present, there is no vaccine available, and Praziquantel (PZQ) which is the chemotherapeutic agent of choice against adult worm, already faces drawback of drug resistance in some *Schistosoma* isolates (WHO, 2001; Mountford, 2005). Sustainable control of schistosomiasis requires integrated approach including repeated mass chemotherapy using praziquantel, public health education focusing on behavior changes towards risk factors, improving sanitation, provision of clean water supply, and snail control using Niclosamide- Bayluscide® (Bayer, Leverkusen, Bayerwerk,Germany) the current synthetic molluscicide used mainly in endemic African countries (Mazigo *et al.,*2012).Since there is a possibility of re-infection even after repeated mass chemotherapy with Praziquantel (Hassan *et al.,*2011), complementing chemotherapy with molluscicide is necessary to prevent re-infection of people in endemic areas.

The high cost of synthetic molluscicides,their negative impacts on the environment including their

toxicity to non-target organisms like fish (Adetunji *et al.,* 2010), as well as fear for emergence of snail resistance to these compounds have given a new impetus to the study of molluscicidal plants (Molla., 2011).Several plants, such as *Phytolacca dodecandra* (Endod), *Solanum xanthocarpum*, *Annona squamosa*, *Thuja orientalis*, *Calotropis procera* and *Adenium arabicum* have already been identified as useful to control the intermediate hosts of trematodes (Karunamoorthi *et al.,*2008; Al-Sarar *et al.,*2012). It is against this backdrop that there has been a resurgence of interest in search for alternative molluscicides of plant origin (Ontarigho *et al.,*2012)

The most potent plant with molluscicidal, cercaricidal and miracicidal activity against snail intermediate hosts of schistosomes and its aquatic larvae is *Phytolacca dodecandra in* Ethiopia (Lemma, 1970). However, it has been shown to be toxic to non-target organisms like fish (Lemma, 1970).Others are *Alternanthera sesselis* (Azare *et al.,*2007), *Balanites aegyptiaca* [Molla,2011] and *Jatropha curcas* (Rupel *et al.,*2000).Plants shown to possess cercaricidal and miracicidal activity include *Entada leptostachya* (Syombua *et al.,*2013), *Bridellia micrantha* (Kindiki *et al.,*2016). Mohamed *et al.,* 2005 reported cercaricidal activity of the crushed seed of *Nigella sativa* to be both time and concentration dependent. Elsewhere cercaricidal and miracicidal studies of *Milletia thoningii* by Perret *et al.,* 1994 and *Irish germanica* by Singaba *et al.,*(2006) also reported the time-concentration relationship of the plants extracts. Any strategy targeting these larval forms of Schistosomes will contribute significantly to control of schistosomiasis.

5.2 MATERIALS AND METHODS

5.2.1 Collection of plant specimen

Six plant species used in this study were collected from their natural habitats on the basis of ethnobotanical information and with bio-conservation aspects in mind. They were from Mavoko location, Machakos County. Taxonomist from the National Museums of Kenya (NMK) authenticated the botanical identity of the plant materials and voucher specimens were preserved in the herbarium. Plant parts collected for use in this study were: leaves of *Annona squamosa* (AS), seeds of *Solanum americanum* (SA), berries of *Solanum linaeanum* (Sl), berries of *Phytolacca dodecandra* (PD),seeds of *Piper nigrum* (PN),and leaves of *Rhizophora mucranata* (Rm) from Kenyan coastal area.

5.2.1 Preparation of plant extracts for miracicidal and cercaricidal bioassay

Plants used in this bioassay was collected as described in 3.2.1.Solvent extract was made by dissolving 25g of each of the fine powdered plant materials in 250 ml of analytical grade methanol overnight with agitation then filtered using muslin cloth and watsman filter paper. Filtrate was extracted in a soxhlet apparatus for 10 cycles at 40 ^{0}C.The extracted material was concentrated to dryness under reduced pressure using rotary evaporator at 45°C to remove methanol.The final extract of each plant was label as PdME,SlME,SaME,AsME,PnME and RmME (i.e. plants initials and ME for methanol extract) then refrigerated till needed for bioassays.

Aqueous extract was prepared by soaking 100 g of each dried plant powder in 1000 ml of distilled water for 24 hours at room temperature (25°C) with shaking. The crude extract was filtered using muslin cloth, and then filtrate frozen and later freeze dried. Yield of each extract recorded after weighing and finally labeled as PdAE, SlAE, SaAE, AsAE, PnAE and RmAE (i.e. AE for aqueous / water extract) then refrigerated till needed for bioassays (Molla ,2011; Salawu *et al.,*2011).

5.2.2 Collection, screening and maintenance of the snail intermediate host

Biomphalaria pfeifferi snails used in this bioassay was collected, screened and maintained as described in 4.2.2 above.

5.2.3 Hatching of miracidia from infected Baboons faeces

Twenty four hour feacal samples were obtained from the chronically infected baboons and transferred into a plastic jug. Water from IPR well with high salt concentration was added, and the mixture stirred with a rod to mix evenly.The mixture was passed through two successive sieves, a 600 µm sieve and a 250 µm sieve into a collecting tray. The collected sample was transferred to urine jars and put in dark room for 30 min.After the 30 min were over, the supernatant was poured out. More water was added to fill up the urine jars and the jars kept in a dark room for a further 30 minutes.This process was repeated four times. After the fourth time, the supernatant was poured out and the pellets transferred using a dropper to already prepared petri dishes containing water and placed under light.The set up was left for a period of 30 minutes for the miracidia to hatch.

5.2.4. Infection of snails and shedding of cercariae

Biomphalaria pfeifferi snails were infected with miracidia that hatched from eggs obtained from faecal samples of olive baboons with chronic *S. mansoni* infection. Three to five miracidia from petri dish placed under dissecting microscope were placed in each well of a 24-well culture plate (Nunclon, Denmark) using a glass pipette mounted on a rubber bulb. One *Biomphalaria pfeifferi* snail was then transferred into each well and the plates covered with their lids to prevent the snails from crawling out and the set up left for 30 minutes for the miracidia to penetrate. The infected snails were then transferred into clean trays with snail water, fed on dry lettuce and the set up maintained for five weeks to allow the development of the miracidia to cercariae (Yole *et al.,* 1996). At the fourth week postinfection, the snails were covered with black cloth to prevent light from

stimulating shedding of cercariae. After five weeks, the infected snails were then carefully removed from tanks using forceps and placed in 10 ml beakers containing snail water. They were illuminated with 100 watts lamp for one hour to allow shedding of cercariae. Cercariae were counted in the 3 aliquotes and average used in bioassays.

5.2.5 Miracicidal activity of plant extracts on miracidia of Schistosoma mansoni .

Only extracts that showed promising molluscicidal activity were tested for miracicidal activity. Different concentrations:10 mg/litre, 20 mg/litre, 50mg/litre and 100 mg/litre and 150mg/l of both the aqueous and the methanol extracts of *Solanum linaeanum* berries*, Solanum americanum* berries, *Phytolacca dodecandra* berries*,Anonna squamosa* leaves *and Piper nigrum* seeds were made. Ten miracidia were placed in 5 cm petri dishes. Two millilitres of each of the already prepared concentrations of the aqueous and ethanol extracts were added to the 10 miracidia in the 5 cm petri dishes. A duplicate was set up for each concentration.Positive control had 2 ml of 1mg/litre niclosamide and negative control had 2 ml distilled water.Each set up was observed under the dissecting microscope, at10, 20, 30 40, 50 and 60 minute intervals for a period of one hour. Dead or immobile miracidia were enumerated and recorded. Constant motion signified that the miracidia were alive while no motion signified death (WHO 1985).

5.2.6 Cercaricidal activity of plant extracts on cercariae of Schistosoma mansoni

Cercariae that was earlier shed from infected snails was used.Aqueous and methanol plant extract concentrations: 10, 20, 50,100 and 150 mg/l (ppm) were prepared. Ten cercariae were placed in a 5 cm petri dish then 2 ml of each of the already prepared concentrations of the extracts were added. Two replicates were used and controls: 2 ml of 1 mg/litre Niclosamide as positive control, 2 ml distilled water as negative control. Dead and surviving cercariae were counted under the dissecting microscope, after every, 10, 20,30,40,50 and 60 minutes. Constant motion signified that the cercariae were alive while dead ones were. Dead cercariae were motionless at the base of petri dish the biferke tails had separated from the cercariae.

5.3 DATA ANALYSIS

Data on miracicidal and cercaricidal activity of plant extracts was analyzed by Finney's probit analysis with the help of BioStat 2009 Version 5.8.4 to estimate LT_{50} (concentration required to kill 50% of miracidia and cercariae) values of the various extracts on miracidia and cercariae. Analysis of Variance (ANOVA) was carried out to compare differences between treatment groups while multiple comparisons between the various treatment groups were done using Turkeys /Dunnet Test and Least Significance Difference Test (LSD).In all the analyses, the probability or significance was at 95% confidence level ($p < 0.05$).

5.4 RESULTS

5.4.1 Mortality of cercariae and miracidia when exposed to extracts

Percentage mortality of miracidia and cercariae of *Schistosoma mansoni* exposed to different extract concentrations at time intervals is shown in Table 4 and Figures 8 and 9 below. *Phytolacca dodecandra* was the most active killing 100% of miracidia after 30minutes and cercariae after 40minutes.*Solanum linaeanum* was next attaining 100% mortality of both miracidia and cercariae after 40 minutes.It took *Solanum americanum* one hour to kill all miracidia and cercariae.The activity of *Anonna squamosa* and *Piper nigrum* was relatively low with only 90% miracidia and 80% cercariae killed after one hour. None of the extracts killed 50% of larvae after 10minutes.For methanol extracts four plants had over 50% larval mortality after 30minutes compared to only two for aqueous extracts.There was increase in mortality as time increased.

Table 9: Miracicidal and cercaricidal activity of extracts on larvae of *Schistosoma mansoni*

	% AVERAGE MORTALITY													
		MIRACIDIA						CERCARIAE						
Aqueous plant Extracts	**5min**	**10min**	**20min**	**30min**	**40min**	**50min**	**60min**	**5min**	**10min**	**20min**	**30min**	**40min**	**50min**	**60min**
Phytolacca dodecandra berries	20	40	80	100	100	100	100	10	30	70	90	100	100	100
Solanum linnaeanum berries	10	20	60	70	100	100	100	10	20	50	80	100	100	100
Solanum americanum berries	0	10	30	40	60	80	100	0	10	20	50	70	90	100
Annona squamosa leaves	0	0	20	40	50	70	90	0	0	10	30	50	60	80
Piper nigrum seeds	0	0	10	30	50	70	80	0	0	0	20	40	60	70
Positive control-Niclosamide	100	100	100	10	100	100	100	100	100	100	10	100	100	100
Negative control-Distilled water	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Methanol extrcts														
Phytolacca dodecandra berries	20	30	70	100	100	100	100	10	30	50	70	90	100	100
Solanum linnaeanum berries	10	30	60	80	100	100	100	10	30	50	70	80	100	100
Solanum americanum berries	0	10	30	60	80	100	100	0	0	20	40	70	100	100
Annona squamosa leaves	0	10	20	50	70	90	100	0	0	10	30	50	60	80
Piper nigrum seeds	0	0	10	20	40	50	70	0	0	0	10	20	40	50
Positive control-Niclosamide	100	100	100	10	100	100	100	100	100	100	10	100	100	100
Negative control-Distilled water	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 33: Cercaricidal activity of plant extracts.

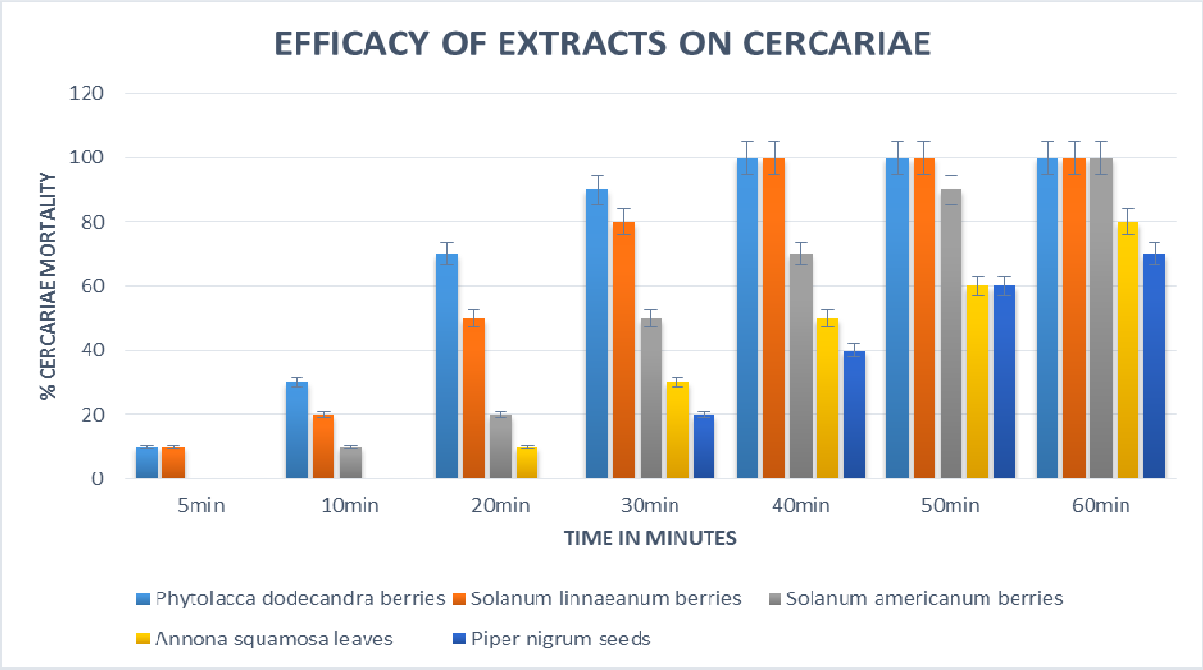
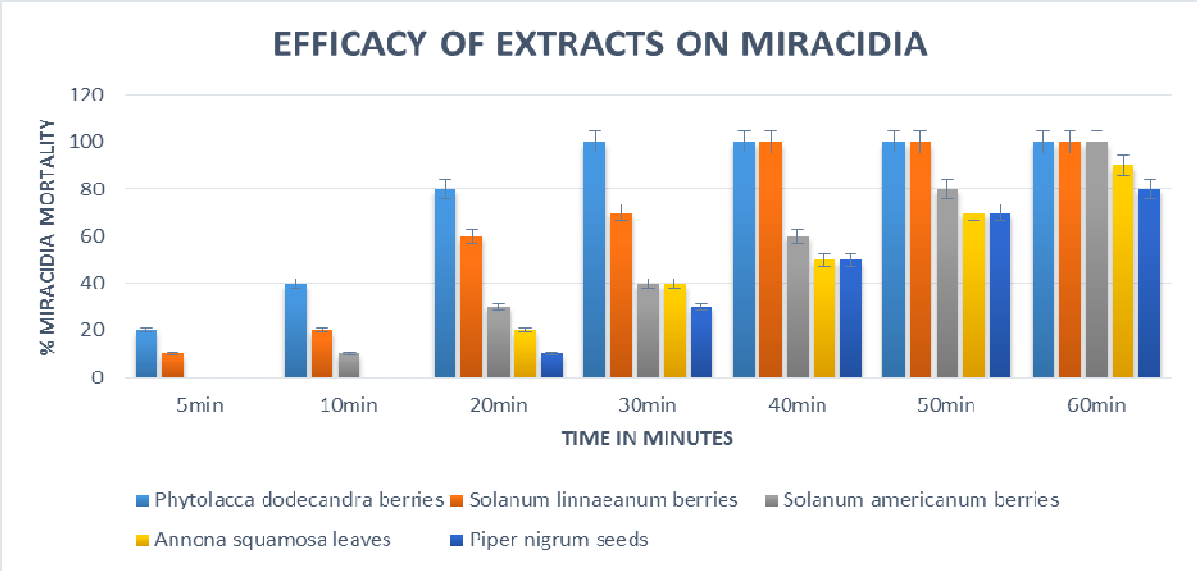


Figure 34: Miracicidal activity of plant extracts.



5.4.2 Determination of lethal time for miracidia and cercariae

Lethal time LT_{50} which is the time required to kill 50% of miracidia and cercariae was determined by probit analysis and results shown in Table 12 and Figures 1 and 2 below.The exposure time required to obtain 50% mortality (LT_{50}) of miracidia and ceraciae decreased with increase in extract concentration. The shortest exposure time required to kill 50% of both miracidia and cercariae at extract concentration of 100ppm was shown by *Phytolacca dodecandra* (LT_{50} 10.84 and 16.94 minutes) and *Solannum linaeanum* (LT_{50} 22.84 and 26.96 min) respectively.This implies that these aqueous extracts had the most active bioactive compounds. However this activity was closely related to that of Niclosamide that recorded LT_{50} 4.75 and 11.50 minutes respectively. Comparison of efficacy of these extracts to Niclosamide showed that there was no significant difference ($p>0.05$) between *Phytolacca dodecandra* and *Solanum linaeanum* meaning that they have miracicidal and cercaricidal activity similar to that of niclosamide the current synthetic molluscicde of choice.

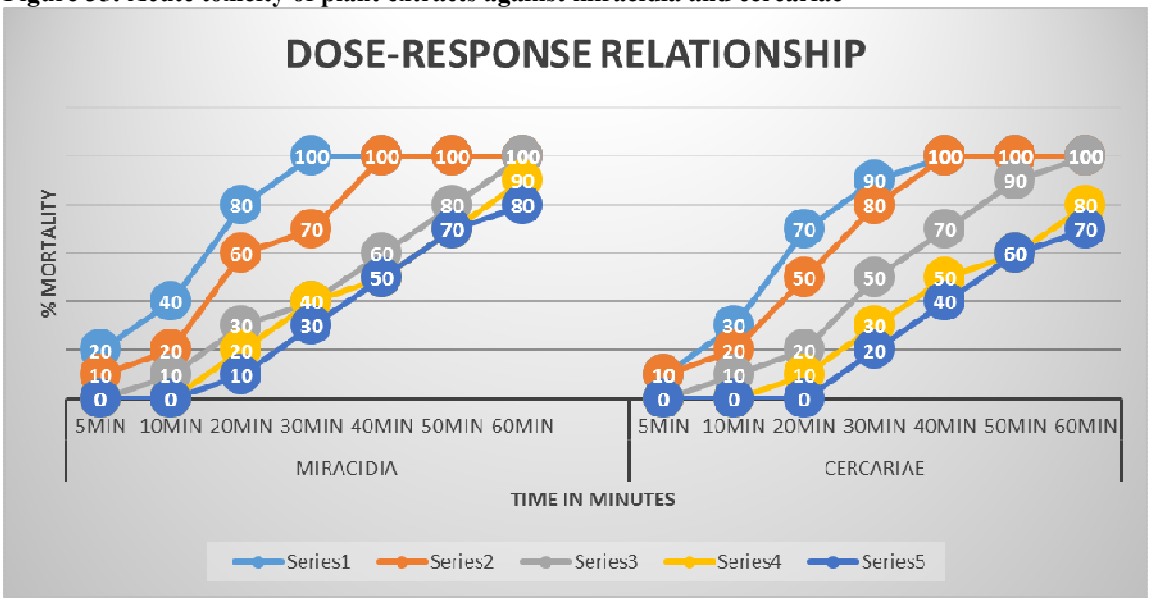
There was no significant difference in percentage mortality of miracidia and cercariae exposed to methanol and aqueous extracts ($p>0.05$).This means that water and methanol were extracting similar bioactive compounds from the plant parts tested in this study.Miracicidal and cercaricidal activity of the rest of the extracts was significantly different ($p < 0.05$).Moderate activity was observed in *Solanum americanum* (LT_{50} 31.02 and 36.89 min) and *Annona squamosa* (LT_{50} 41.29 and 46.35 min).The longest exposure time required to obtain 50% larval mortality was shown by *Piper nigrum* (LT_{50} 52.84 and 58.30 min.) respectively.This means that it possessed the leased active compounds.There was a significant difference in susceptibility of miracidia and cercariae to extracts ($p < 0.05$).Miracidia was more sensitive to all extracts tested than cercariae.

Table 10: Exposure time required to obtain LT_{50} mortality of miracidia and cercaria.

EXTRACT	No.of larvae used	Miracicidal LT_{50} ± SE	Cercaricidal LT_{50}±SE
Phytolacca dodecandra	10	10.84 ± 3.42	16.91 ± 3.59
Solanum linaenum	10	22.84 ± 4.31	26.96 ± 3.59
Solanum americanum	10	31.02 ± .51	36.89 ± 5.38
Anonna squamosa	10	41.29 ± 4.94	46.35 ± 4.94
Piper nigrum	10	52.84 ± 3.42	58.30 ± 12.47
Niclosamide	10	4.75±2.50	11.50±2.65
Distilled water	10	0	0

Alpha $p < 0.05$, SE=Standard error, LT=Lethal time.

Figure 35: Acute toxicity of plant extracts against miracidia and cercariae



KEY: 1-*Phytolacca dodecandra*, 2-*Solanum linaeanum*, 3-*Solanum americanum*, 4-*Annona squamosa*, 5-*Piper nigrum*

5.5 DISCUSSION

Sustainable control of schistosomiasis requires that the life cycle of Schistosomes should be interrupted by killing cercaiae and miracidia. Niclosamide, the synthetic molluscicde currently in use to control the aquatic host snails, miracidia and cercariae, is toxic to non-target organisms like fish, very expensive and resistance of snails is highly probable. Search for biorationals that can decrease the shedding of cercariae and kill both cercariae and miracidia is important.The current study sought to evaluate the cercaricidal and molluscicidal potency of Kenyan *Phytolacca dodecandra, Solanum linaeanum, Solanum americanum, Anonna squamosa* and *Piper nigrum*.A part from *Phytolacca dodecandra* whose activity has been reported in Ethiopia,the rest are being reported for the first time.Probit analysis of acute toxicity of plant extracts against miracidia and cercariae of *Schistosoma mansoni* showed that *Phytolacca dodecandra and Solannum linnaeanum* were the most active registering the shortest exposure time (LT_{50} 10.84 and16.91min); LT_{50} of 22.86 and 26.96min) respectively. This means that these extracts killed 50% of miracidia and cercariae within 10, 16; 22 and 26 minutes respectively.There was no significant difference in larval mortality between these extracts and niclosamide implying that these two plant species have cercericidal and miracicidal activity similar to that of the currently used synthetic molluscicide.*Solanum americanum* and *Annona squamosa* showed moderate activity killing miracidia and cercariae after 30 and 40 minutes respectively.*Piper nigrum* was observed to have the longest exposure time (LT_{50} 52.84 and 58.30 min) implying it had the least active compounds against miracidia and cercariae respectively. According to Akillu *et al.,*(1978),the concentration of endod and niclosamide which kills tilapia species are same as that needed to kill snails hence *Phytolacca* dodecandra is toxic to non –target organisms.

The study also suggested the possibility that endod applied to natural water bodies in concentrations sufficient to kill snails would kill cercariae and miracidia there by contributing to the control schistosomiasis.This is in line with findings of this study that revealed that cercariae and miracidia were susceptible to extract concentrations similar to those used on molluscicdal assay by the same author.Even though both water and methanol extracts were used in these bioassays, there was no significant difference in percentage mortality of miracidia and cercariae exposed to methanol and aqueous extracts ($p>0.05$).This means that water and methanol were extracting similar bioactive compounds from the plant parts tested in this study.The first criterion in the assesment of plant molluscicides, cercaricides and miracicides is that it should be polar.Such a property enhances biodegradability and reduces possible built up / accumulation of toxic residues in the Environment.Production of biorationals by an inexpensive and efficient method would be helpful to communities in endemic areas. There was a significant difference in susceptibility of miracidia and cercariae to extracts ($p < 0.05$).Miracidia were found to be more sensitive than cercariae to the lethal effect of the extracts.This high susceptibility has also been reported by other researchers: Kindiki *et al.,* 2016;Syombua *et.al*., 2013.The observed higher activity of the extracts against miracidia is advantageous since killing one miracidium prevents the formation of thousands of cercariae which would contaminate the water and infect the people who get in contact with such water bodies. All the four efficacious extracts killed Schistosome larvae at concentrations less than 100ppm.

5.6 CONCLUSSION

From the present study, it can be concluded that both aqueous and methanol solvents extracted similar compounds from plants tested and exhibited significantly similar toxicity to schistosome larvae. The most efficacious extracts were polar meaning the active principles can be extracted by water hence better alternative miracicidal and cercaricidal agent. Miracidia were more susceptible to toxic effects of extracts than cercariae. *Phytolacca dodeccandra* and *Solanum linaeanum* were the most efficacious against both miracidia and cercariae of *Schistosoma mansoni*. This activity was similar to that of niclosamide the mollucicide currently in use for control of host snails. Therefore these plants possess potent, polar bioactive compounds that could be developed into miracicide and cercaricide thereby offering cheap, effective and environmentally friendly alternative for control of Schistosomiasis in endemic areas.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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