

Assessment of Molluscicidal, Cercericidal and Miracidal Activities of Crude Extracts of *Azadirachta indica* and *Entada leptostachya*

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Abstract

Schistosomiasis infections in humans depend absolutely on the presence of intermediate host. Control of the intermediate host disrupts the cycle of schistosomes stopping transmission of schistosomiasis. Synthetic molluscicides used today are expensive and toxic to non-target organisms. Herbal preparations which do not affect non-target organisms would be a key in controlling schistosomiasis. This study was done to determine if plant extracts of *Entada leptostachya* and *Azadirachta indica* exhibit molluscicidal, cercericidal and miracidal activities. *Biomphalaria pfeifferi* adult snails and juveniles, *Schistosoma mansoni* cerceriae and miracidia were used in the study. Groups of uninfected snails were exposed to different concentrations of water, methanol and ethyl acetate crude extracts obtained from the two plants. Controls were also set; positive control (Niclosamide) and negative control (Distilled water). Miracidia and cerceriae were exposed to the most active plant extract on juvenile and adult snails. Data analysis was done using Finney probit analysis to estimate the LD₅₀ values of the crude extracts on snails and LT₅₀ values of the crude extracts on cerceriae and miracidia. Only methanol extract of *E. leptostachya* was found to exhibit the highest molluscicidal activity on juveniles and adults with a LD₅₀ value of 30.21 mg/l and 40.93 mg/l respectively ($P \leq 0.05$). Methanol extract of *A. indica*, aqueous and ethyl acetate extracts of *A. indica* and *E. leptostachya* were nontoxic to both adult and juvenile snails. On the other hand, methanol extract of *E. leptostachya* were found to have cercericidal and miracidal activity. The LT₅₀ of miracidia and cerceriae was 7.69 minutes and 4.25 minutes respectively at a concentration 80 mg/l ($P \leq 0.05$). Phytochemical screening of the methanol, aqueous extracts and ethyl acetate extracts of *A. indica* and *E. leptostachya* confirmed the presence of flavonoids, saponins, tannins, alkaloids, triterpenes and sterols. Results suggest that methanolic root extract of *E. leptostachya* has molluscicidal activity against *Biomphalaria pfeifferi*. The results also indicate that methanolic root extract of *E. leptostachya* have cercaricidal and miracidal activity against the schistosome larval stages.

Keywords: *Azadirachta indica*, *Entada leptostachya*, *Biomphalaria pfeifferi*, Crude extracts, Phytochemical screening, Molluscicidal activity, Cercericidal activity and Miracidal activity

1. Introduction

Human schistosomiasis is a parasitic disease caused by digenetic trematode species of the genus *Schistosoma* which co-habits the venous plexuses of the mammalian viscera (Lockyer *et al.*, 2003). It is a major public health problem in tropical and subtropical regions of the world where an estimated 200 million people are infected and close to a billion people are at risk of contracting the disease (Gollin & Zimmermann, 2010). It occurs in over 70 countries of the tropic and sub tropics (David *et al.*, 2006) causing an estimated 500,000 deaths every year (WHO, 2001) and it is the second most devastating parasitic disease after malaria (WHO, 1993). It is one of the most prevalent parasitic infections and has significant economic and public health consequences (Chitsulo *et al.*, 2000). Schistosomiasis infections in humans depend absolutely on the presence of intermediate host the snail. *Biomphalaria pfeifferi* is the snail which transmits *Schistosoma mansoni*. *Schistosoma mansoni* eggs from faeces of infected snails develop into miracidia. Miracidia go through developmental stages in snail to become cerceriae which are released by the snails. Humans get infected when they enter cerceriae infested waters for domestic, occupational and recreational purposes. Synthetic molluscicides used today have some drawbacks. The commonly used molluscicide Niclosamide, is

effective but to be able to achieve best results; the application has to be done at least twice a year. This is not affordable by the local communities (Oketch *et al.*, 1998). According to WHO (1965) Niclosamide (Bayluscide) is highly toxic to snails and eggs but difficult to formulate and it also targets other organisms like the fish. As a result, plants have been frequently studied as alternative sources to chemical molluscicides. A number of substances of vegetable origin have been found to have molluscicidal activity.

Dried berries of *Phytolacca dodecandra* (Phytolaccaceae) are widely used in Ethiopia instead of soap for washing clothes. It was observed that in natural bodies of water where *P. dodecandra* had been used, there was a high mortality of snails. *P. dodecandra*, the best studied plant molluscicide to date, is commonly used to treat intestinal worms in Ethiopia (Esser *et al.*, 2003). It stands out clearly having been well researched up to field trial stages, of which a lot has been published through the work of Lema's group (Lema, 1965; Lambert *et al.*, 1991). The molluscicidal effects of various parts of the plant have been determined, and the berries were found to have the greatest molluscicidal activity. Others include plant species of the genus *Solanum* (Solanaceae), are reported to show a variety of activity attributed to presence of saponins or steroidal alkaloids or both. The LC₅₀, and LC₉₀ for leaf extracts of *Solanum nigrum*, *Solanum sinaicum*, and *Solanum villosum* revealed that ethanol extract of *S. nigrum* leaves showed the highest molluscicidal activity (LC₉₀ = 5.95 mg/l), followed by *S. sinaicum* (6.04 mg/l) and *S. villosum* (8.95 mg/l) (El-Sherbini *et al.*, 2009). In this study, plant extracts of two medicinal plants; *Entada leptostachya* (roots) and *Azadirachta indica* (leaves) used in different parts Kenya in the treatment of various ailments were investigated for molluscicidal activity against *Biomphalaria pfeifferi*. They were also investigated for their cercericidal and miracidicidal activities against *Schistosoma mansoni* larval stages.

2. Materials and Methods

2.1. Collection, Drying of Plants and Identification

Two plants parts; leaves of *Azadirachta indica* A.Juss. (Meliaceae) and roots of *Entada leptostachya* Harms. (Leguminosae) were collected from Mbeere District, Eastern Kenya and stored in plastic bags and transported to the laboratory for processing. The plants parts were dried at room temperature for one month. They were crushed into tiny particles Using Mekon Micro miller Single phase and passed through a 0.5 mm mesh to standardize the particles. The powder was then stored in screw topped glass bottles and kept at room temperature. Aqueous, methanol and ethyl acetate extraction were carried out using the powder for the two plants. 1 kg of the ground powder of each of the extract was extracted with a considerable amount of solvent/distilled water. The organic extracts were concentrated in a rotary evaporator while the aqueous extracts were freeze-dried into a thick a gummy extract.

2.2. Phytochemical Screening

Phytochemical screening was performed using standard procedures (Harborne, 1998; Siddiqui and Ali, 1997; Karumi *et al.*, 2004; Ayoola *et al.*, 2008). The various extracts were tested for triterpenes, sterols, flavonoids, saponins, tannins and alkaloids and glycosides.

2.3. Maintenance of the Snail Host in the Laboratory

The snails were collected from Mwea irrigation scheme, Eastern Kenya. They were screened for schistosomes under strong light (100watts) daily for 5 weeks. Those that were negative for cerceriae were maintained at the Institute of Primate Research (IPR) snail laboratory. The snails were housed in a temperature controlled snail room (25 – 28°C) in plastic tanks layered with sterilized sand and gravel from Mwea. They were fed on dried lettuce and daphnia was used for aeration.

2.4. Infection of Snails with *S. mansoni*

Snails were infected with miracidia from eggs of chronically infected baboons maintained at IPR Animal Resource Department. The molluscicidal activity against mature adult and juvenile snails was conducted in accordance with WHO (1965) guidelines. Groups of ten uninfected adult snails were transferred to transparent plastic containers with distilled water and left for 24 hrs. Distilled water was replaced with specified concentrations (1 mg/l, 5 mg/l, 10 mg/l, 15 mg/l, 20 mg/l, 40 mg/l and 80 mg/l) of the 3 extracts from the two plants. Duplicates were set up for each concentration. Duplicates were set for controls, distilled water and Niclosamide (1 mg/l). After exposure to the extracts the snails were given a recovery period of 24 hrs in distilled water. Snail mortality was recorded from each set up. Dead snails remained retracted inside their shells at the bottom of the plastic container and failed to show any response to mechanical stimulation with a wooden spatula. Death was confirmed by lack of heart beat.

2.5. Cercericidal Activity

The plant extracts which had shown molluscicidal activities were selected for cercericidal work. Two milliliter of (1 mg/l, 5 mg/l, 10 mg/l, 15 mg/l, 20 mg/l, 40 mg/l and 80 mg/l) *E. leptostachya* was dispensed in each well of the 24 well culture plates containing 10 cerceriae. Two replicates for each concentration were made. Each preparation was observed under a dissecting microscope for cerceriae motility at the following time points: 5, 15, 30 and 60 minutes. Immobile cerceriae were enumerated and recorded at every time point.

2.6. Miracicidal Activity

The plant extracts which had shown molluscicidal activities were selected for miracicidal work. Two milliliter of (1 mg/l, 5 mg/l, 10 mg/l, 15 mg/l, 20 mg/l, 40 mg/l and 80 mg/l) *E. leptostachya* was dispensed in each well of the 24 well culture plates containing 10 miracidia and then observed under a dissecting microscope for miracidia motility at the following time points: 5, 10, 20, 30, 40 and 60 minutes. Immobile miracidia were enumerated and recorded at every time point.

2.7. Data Analysis

Data was analysed by Finney's probit analysis with the help of BioStat2009 Version 5.8.4 to estimate LD₅₀ (concentration required to kill 50% of the juvenile and adult snails) values of the various crude plant extracts on adults and juvenile snails. Finney's probit analysis was also used to estimate LT₅₀ (exposure time required to kill 50% of the miracidia and cerceriae) values when the miracidia and cerceriae were exposed to various concentrations of the methanolic root extract of *E. leptostachya* which was found to be the most active extract against juvenile and adult snails

3. Results

3.1. Mortality of Juvenile and Adult snails

Table 1 below shows the average mortality of both juvenile and adult snails when subjected to different concentrations of the various concentrations of *A. indica* and *E. leptostachya* aqueous and organic extracts.

Table 1. Percentage average mortality of juvenile and adult snails

Concentration (mg/l)	Percentage Average Mortality											
	<i>A. indica</i>						<i>E. leptostachya</i>					
	Juvenile			Adults			Juvenile			Adults		
	MOH	EAc	Aq	MOH	EAc	Aq	MOH	EAc	Aq.	MOH	EAc	Aq
1	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
10	5	0	10	0	0	0	0	5	0	5	0	5
20	5	5	0	5	0	5	20	0	5	5	5	5
40	5	5	0	5	0	0	75	0	5	60	0	5
80	5	15	0	15	10	5	100	0	15	95	5	5
Controls	Juveniles						Adults					
Niclosamide (1mg/l)	100						100					
Distilled Water	0						0					

EAc = Ethylacetate extract; MOH= Methanol extract; Aq= Aqueous extract

Methanolic plant extracts of *E. leptostachya* recorded an average percentage mortality of to 20%, 75%, and 100% on juvenile snails for 20, 40 and 80 mg/l respectively. Similarly, the methanolic plant extracts of *E. leptostachya* recorded a high percentage mortality of to 60% and 95% mortality on adult snails for both 40 and 80mg/l respectively. There was no retraction at all in the dead snails to mechanical stimulation of the foot-sole with a wooden spatula which was confirmed by absence of heart beat. This was close to what was observed in positive control (1 mg/l Niclosamide) for both the first and the duplicate set up. In all the other tested plant extracts (methanol, ethyl acetate and aqueous of *A. indica*, ethyl acetate and aqueous extracts of *E. leptostachya*), the results were similar to what was obtained with the distilled water (negative control), where either none or just one snail died in the duplicate set ups. This translated to either 0% or 5% mortality.

3.2. Acute toxicity of the Crude Extracts

LD₅₀ was estimated using Finney's probit analysis and the results are tabulated in Table 2.

Table 2: Acute toxicity of the crude plant extracts

Extract	Plant species	Juvenile snails (LD ₅₀ ±SE)(mg/l)	Adult snails (LD ₅₀ ±SE)(mg/l)
Aqueous extract	<i>A. indica</i>	2,000.21±4.043	4,397.20±5.178
	<i>E. leptostachya</i>	1560.64±8.646	3678.90±11.36
Methanol extract	<i>A. indica</i>	3678.90±11.36	1560.64±8.646
	<i>E. leptostachya</i>	30.21±4.59	40.93±2.75
Ethyl acetate extract	<i>A. indica</i>	1560.64±8.646	2,000.21±4.043
	<i>E. leptostachya</i>	6981.93±2.23	4397.20±5.178

Alpha (P) ≤ 0.05, SE = Standard error

A plant extract with a LD₅₀ ranging between 0-500 mg/l indicates that the extract is highly toxic; a plant extract with a LD₅₀ ranging between 500-1000 mg/l indicates that the extract is moderately toxic while a plant extract with a LD₅₀ which is over 1000 mg/l indicates that the plant is non toxic (Nguta *et al.*, 2011). The methanol extract of *E. leptostachya* were the most active plant extracts with a LD₅₀ of 30.12 mg/l and 40.93 mg/l against juvenile and adult snails respectively. All the other extracts (methanol, ethyl acetate, aqueous of *A. indica*, ethyl acetate and aqueous extracts of *E. leptostachya*) had LD₅₀ of < 1000 and therefore non toxic.

3.3. Cercerical and Miracidial Activity

Miracidial and Cercarial LT₅₀ were estimated by Finney's probit analysis. The results are tabulated below.

Table 3: Exposure time required to obtain 50% miracidia mortality and 50% cerceriae mortality

Concentration(mg/l) of methanol extract of <i>E. leptostachya</i>	N (total number of Miracidia /Cercaria used)	Miracidial LT ₅₀ ±SE (minutes)	Cercarial LT ₅₀ ±SE (minutes)
1	10	41.72±5.28	38.40±8.67
5	10	38.31±5.39	24.09±8.95
10	10	19.07±2.34	14.74±4.75
20	10	15.38±1.48	8.79±2.51
40	10	7.69±0.96	4.29±4.48
80	10	7.69±0.96	4.25±2.13
Niclosamide (1mg/l)	10	5.46±1.42	11.19±3.44

Alpha (P) ≤ 0.05, SE = Standard error

E. leptostachya methanolic extract which was found to have molluscicidal activity on both adults and juveniles *B. Pfeifferi* was used for the *in vitro* miracidial and cercarial activity. The exposure time required to obtain 50% mortality (LT₅₀) of miracidia decreased with increase in concentration of the methanolic extract of the root of *E. leptostachya*. At 40 mg/l and 80 mg/l the LT₅₀ was 7.69 minutes implying that these concentrations of the root extracts of *E. leptostachya* are highly active against miracidia larvae. At 1 mg/l and 5 mg/l concentration of the *E. leptostachya* methanol extract, the miracidial LT₅₀ was 41.72 minutes and 38.31 minutes respectively. Hence more time elapsed in obtaining 50 % miracidial mortality at low concentrations. This implies that these low concentrations of the extracts were less active on the miracidia. On the other hand, the exposure time required to obtain 50% mortality of cerceriae shortened with increase in concentration of the methanol extracts of the root of *E. leptostachya*. The shortest exposure time causing 50 % cerceriae mortality was observed at concentration of 80 mg/l of the *E. leptostachya* methanol root extract which was 4.25 minutes. This LT₅₀ of 4.25 minutes was far much shorter than LT₅₀ of 11.19 minutes observed when the Niclosamide was used. On the contrary, the longest exposure time required to obtain 50 % cerceriae mortality was observed at 1mg/l of *E. leptostachya* methanol root extract which was 38.40 minutes.

3.4. Phytochemical Screening Results

Phytochemical screening of the crude plants extracts revealed some differences in the constituents of the *E. leptostachya* and *A. indica*. Saponins, triterpenes, glycosides, flavonoids, alkaloids and tannins were detected in methanol, aqueous and ethyl acetate crude extracts of *A. indica* (leaves) and *E. leptostachya* (roots). However tannins

and alkaloids were absent in ethyl acetate extract of *A. indica* and *E. leptostachya* respectively. Results are tabulated in the Table 4.

Table 4. Phytochemical constituents present in the crude extracts

Classes of phytochemical constituents	<i>Azadirachta indica</i> (leaf extract)			<i>Entada leptostachya</i> (roots extract)		
	EAc	MOH	Aq	EAc	MOH	Aq
Alkaloids	+	+	+	-	+	+
Saponins	+	+	+	+	+	+
Tannins	-	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Sterols/Triterpenes	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+

+ =Present; - = Absent; EAc = Ethylacetate extract; MOH= Methanol extract; Aq= Aqueous extract

4. Discussion

4.1. Molluscicidal Effects

From the results methanol extract of *E. leptostachya* roots had the highest molluscicidal activity against adults *B. pfeifferi* snails. At 40mg/l and at 80mg/l concentration of the extracts, 60% and 95% mortality of adult snails was observed respectively. This was not significantly different from the mortality caused by Niclosamide which was 100%. The LD₅₀ value of the methanol root extract of *E. leptostachya* was 40.93 mg/l implying that the extracts were highly active against *B. pfeifferi* adult snails. On the other hand no molluscicidal activity was observed in aqueous extracts of *E. leptostachya* and ethyl acetate extracts *E. leptostachya* on adult snails both with a LD₅₀ value > 1000 mg/l. Similarly aqueous leaf extracts, methanol leaf extracts and ethyl acetate extracts of *A. indica*, with a LD₅₀ ranging above 1000 mg/l on adult snails were also considered as non toxic to adult *B. pfeifferi* snails. High molluscicidal activity was observed in the methanol extract of *E. leptostachya* on juvenile snails. At 40mg/l and at 80mg/l concentration of the extracts, 75% and 100% mortality of juvenile snails was observed respectively which was also similar to mortality caused by Niclosamide. The estimated LD₅₀ value of the methanolic root extract of *E. leptostachya* on the juvenile snails was 30.21 mg/l. This shows that the extracts were more active on the juvenile snails than on the adult snails (LD₅₀ of the extracts on adult snails was 40.93 mg/l). This can be attributed to the fact that juvenile snails do not have well developed protective mechanism hence more of them succumbed to the extracts. *E. leptostachya* ethyl acetate and aqueous extracts had a LD₅₀ value of 6981.93 mg/l and 1560.64 mg/l on juvenile snails respectively and hence were non toxic to juvenile snails. Aqueous, methanol and ethyl acetate leaf extracts of *A. indica* with a LD₅₀ value of 2000.21 mg/l, 3678.90 mg/l, and 1560.64 mg/l were non toxic on *B. pfeifferi* juvenile snails. Therefore, the most active extract with similar activity like niclosamide was the methanolic extracts of *E. leptostachya*. Yasuraoka *et al.* (1977) while investigating the molluscicidal activity of a related species *Entada phaseoloides* (Leguminosae) found out that the methanolic bark extract of the plant were active against *Oncomelania quadrasi* with the LC₅₀ of 3.6-5.8 ppm and this supports the results obtained in this study of molluscicidal activity methanolic root extract of *E. leptostachya*. Other studies on the molluscicidal activities of *A. indica* root, leaf and bark crude extracts showed that the extracts were less toxic on two land snails *Archachatina marginata* and *Limicolaria aurora* and mortality was only witnessed after 48 and 72 hours of exposure at high concentrations of 500 mg/kg and 700 mg/kg (Ebenso, 2003). This is in line with the results obtained in this study on the molluscicidal activity of *A. indica* that showed non toxicity of leaf extracts of *A. indica* on *B. pfeifferi* snails.

Plants have various bioactivities which include molluscicidal, antifungal, antibacterial, larvicidal, cytotoxic and insecticidal besides others (Norman, 1966). Biological activities are due to the presence of various secondary metabolites present in the plants (Norman, 1966). The phytochemicals are concentrated in various parts of the plant while others are equally distributed in all parts of the plant. Results of phytochemical screening of *A. indica* leaf and *E. leptostachya* root extracts indicate that flavonoids, sterols, terpenes, glycosides, saponins, alkaloids and tannins were present in aqueous, methanol and ethyl acetate extracts of *A. indica* and *E. leptostachya* except alkaloids and tannins were absent in ethyl acetate extracts of *E. leptostachya* and *A. indica* respectively. Several alkaloids belonging to pyridine group with molluscicidal activity have been isolated from the bark and stem of *Punica*

granatum Linn. Punicaceae which have been shown to be active against *Lymnaea acuminata* (Tripathi & Singh, 2000). Elsewhere flavonoids, coumarins, triterpenes, saponins and alkaloids with molluscicidal activity have been identified in *Chenopodium ambrosioides* L.(Chenopodiaceae) and *Ruta chalepensis* L. (Rutaceae) (Hmomouch *et al.*, 2000). According to Singh (1998) a toxic glycoside isolated from the stem bark of *Nerium indicum* Mill. (Apocynaceae) is responsible for the toxicity of the bark extract against *Lymnaea acuminata*.

From the above information, secondary metabolites belonging to the major classes identified in *A. indica* and *E. leptostachya* extracts have been shown to have molluscicidal activities and therefore they could be the active compounds which acted against both adult and juvenile *B. pfeifferi* snails, miracidia and cercaria as it was observed in this study. Indeed methanolic extract of *E. leptostachya* had alkaloids, saponins, tannins, flavonoids, sterols, triterpenes, glycosides hence the observed high molluscicidal activity in the present study could be attributed to the presence of these secondary metabolites which could be present in high concentrations. Although the secondary metabolites were present in both active and non active extracts, most probably the critical concentration threshold had not been attained in the non active forms.

4.2. Cercericidal and Miracidal Effects

Cercaricidal and miracidal activity of the active extract: methanolic root extract of *E. leptostachya* showed that when cercariae were exposed to high concentrations of the extract, high mortality was witnessed within a short time. At the 15th minute all the cercariae were dead at concentration 80mg/l while at 30th minute all the cercariae were dead at the concentration of 40mg/l. The exposure time required to obtain 50 % cercarial mortality (LT₅₀) at 40 mg/l and at 80mg/l of the methanol extract of *E. leptostachya* was 4.29 minutes and 4.25 minutes respectively. This exposure time was far shorter than the LT₅₀ for Niclosamide (1 mg/l) which was 11.19 minutes. This can be explained by the fact that the *E. leptostachya* methanolic root extract could have had a high concentration of the active phytoconstituents which could be responsible for their high toxicity causing a shorter LT₅₀. At the 10th minute all the 10 miracidia were dead at 40mg/l and 80mg/l concentration. The exposure time required to obtain 50% miracidal mortality at 40mg/l and 80mg/l was 7.69 minutes. In comparison with the time required to obtain 50% cercarial mortality at 40 mg/l and 80mg/l which was 4.29 minutes and 4.25 minute respectively shows that the time required to obtain 50% cercarial mortality was shorter than that required to obtain 50% miracidal mortality at the different concentrations. This implies that the cercariae were more susceptible to the methanolic extracts of the root of *E. leptostachya* which could be attributed to the fact that they do not have well developed protective structures compared to miracidia which are encased in a protective calcareous shell. Cercariae are the infective larval stages of schistosomes and are specifically adapted to swimming and penetration of the human skin.

5. Conclusion and Recommendations

E. leptostachya methanolic root extract was shown to have high molluscicidal activity while the extracts from *A. indica* were non toxic to *B. pfeifferi* snails. Methanolic root extract of *E. leptostachya* was more active against juvenile snails than adult snails and at a concentration 80 mg/l, the extract had a molluscicidal activity which was not significantly different from that of Niclosamide. In addition to molluscicidal activity, *E. leptostachya* methanol root extract has also been shown to have cercericidal and miracidal activity with the extract being more active against cercaria than miracidia. Phytochemical screening showed that alkaloids, saponins, triterpenes, flavonoids, tannins, glycosides and sterols were present in the active extract and could be the potent molluscicidal agents. Further phytochemical analysis of the methanol root extract of *E.leptostachya* is needed. This will involve isolation of the various phytochemical constituents which will then be investigated for their molluscicidal activities, miracidal and cercaricidal activities. This can be one move in the search for new molluscicides in the process of combating schistosomiasis.

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