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Ovicidal effect of ethanolic extracts of selected plants on eggs of liver flukes *in vitro*

M. AHMED[1],*, A. ELAMIN[2], M. BAHA SAEED[2], M. LAING[2]

[1,]*Department of Medical Laboratories, College of Applied Medical Sciences, University of Qassim, Almulaida 52571, Saudi Arabia, E-mail: *maw.ahmed@qu.edu.sa*, *mawahibalhag@gmail.com*; [2]Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Science, University of KwaZulu-Natal, Pietermaritzburg 3200, South Africa, E-mail: *amalelamin@gmail.com*, *mohamedbaha75@gmail.com*, *laing@ukzn.ac.za*

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Summary

Fasciolosis is a parasitic disease that affects both humans and animals. Due to parasitic resistance to chemical drugs, there has been a growing focus on studying the anthelmintic properties of plants. *In vitro*, studies were conducted on the ovicidal activity of ethanolic extracts of 29 plants against eggs of liver flukes from cattle. Plants were selected due to their availability and previous literature reports. Each plant's ethanolic extract was tested at a concentration of 20 % of the raw extract. The incubation period was 15 days at 28°C for all treated eggs, while control samples were treated with ethanol and water. Mortality levels of eggs ranged from 0 to 100 %. *Moringa oleifera, Ananas comosus, and Foeniculum vulgare* caused the highest mortality levels of 100 %, 100 %, and 90 %, respectively, followed by *Cymbopogon nardus* and *Artemisia afra*, which caused mortality levels of 62 % and 60 %, respectively. The plant extracts were then used in a concentration-response experiment using 5 %, 10 %, and 20 % extracts. Extracts from *Moringa oleifera* and *Ananas comosus* showed the highest ovicidal activity at the three concentrations, followed by *F. vulgare*, *C. nardus*, and *A. afra*. At the 5 % concentration, *M. oleifera* and *A. comosus* extracts were both ovicidal, with rates of 83 % and 80 %, respectively.

Keywords: Anthelmintics; Parasitology; Liver flukes; Plant extracts; Efficacy

Introduction

Fasciolosis is a parasite infection that affects both livestock and human health globally (McIlroy *et al*., 1990; Spithill *et al*., 1999; Lalor *et al*., 2021). The primary flukes that induce clinical infection are *Fasciola hepatica* (Linnaeus) and *Fasciola gigantica* (Cobbold) (Mas-Coma *et al*., 2007). Fasciolosis symptoms in animals include weight loss (Elitok *et al*., 2006), decreased milk and wool production (Schweizer *et al*., 2005; Charlier *et al*., 2007), fertility problems (Loyacano *et al*., 2002), liver necrosis (Daryani *et al*., 2014; Marcos *et al*., 2007), and death in severe infections (McIlroy *et al*., 1990; Rapsch *et al*., 2008). Fascioliasis reportedly causes livestock losses of over $3 billion annually worldwide (Mungube *et al*., 2006; McGonigle *et al*., 2008; Utrera-Quintana *et al*., 2022). In humans, pathogenesis can result from immature migratory flukes injuring the liver parenchyma and generating abscesses in blood vessels, stomach pain, fever, painful hepatomegaly, ascites, and jaundice (Moazeni *et al*., 2017). In addition, inflammation in the bile ducts leads to fibrosis (Tanwar *et al*., 2020).

Anthelmintic medications are commonly used to treat fasciolosis (Fairweather & Boray, 1999; López-Abán *et al*., 2007). Triclabendazole is the most regularly used medicine for controlling flukes (Boray *et al*., 1983; Keiser *et al*., 2005). However, the continued use of triclabendazole has resulted in fluke resistance, an issue

* – corresponding author

that has been recorded in many regions of the world (Overend & Bowen, 1995; Moll *et al*., 2000; Olaechea *et al*., 2011; Daniel *et al*., 2012; Gordon *et al*., 2012; Hanna *et al*., 2015; Melinda *et al*., 2024). Thus, alternate fluke control measures are required. Plant extracts offer interesting alternatives to pharmaceutical control for liver flukes (Haçarız *et al*., 2009).

The majority of plant extract research has focused on suppressing adult flukes. *Acacia Senegal* (Alsadeg *et al*., 2015), *Allium sativum*, *Ferula assa-foetida*, *Syzygium aromaticum, Lawsonia inermis*, and *Opuntia ficus-indica* (Jeyathilakan *et al*., 2012) have all been evaluated in this position. However, controlling liver fluke eggs to prevent miracidia development or hatchability would be beneficial in reducing the spread of these parasites (McManus, 2020). Pereira *et al*. (2016) noted that there has been minimal research into the biocontrol of liver fluke eggs. This is the only study we know that has evaluated the ovicidal efficacy of 29 selected plants from various families against liver fluke eggs from cattle in Southern Africa.

Material and Methods

Plant materials

Twenty-nine plant samples (Table 1) were selected based on previous reports of their bioactivity against parasites (Ahmed *et al*., 2012; Fomum, 2018). Nine were collected from the University of KwaZulu-Natal (UKZN) Botanical Garden, Pietermaritzburg (KwaZulu-Natal Province), latitude 29.37 S, longitude 30.24 E, 655 meters above sea level. Eighteen plants were collected from

Table 1. List of plant species and the organs that were evaluated for their anthelmintic activity.

No	Family Name	Scientific Name	Common Name	Organs Used
1.	Fabaceae	*Acacia karoo*	Sweet thorn	Leaves and stem
2.	Fabaceae	*Acacia tortilis*	Umbrella thorn	Leaves and stem
3.	Asteraceae	*Achillea millefolium*	Yarrow	Leaves
4.	Liliaceae	*Allium sativum*	Garlic	Bulbs
5.	Asphodelaceae	*Aloe ferox*	Bitter aloe	Stem
6.	Bromeliaceae	*Ananas comosus*	Pineapple	Leaves
7.	Papaveraceae	*Argemone mexicana*	Mexican prickly poppy	Leaves and stem
8.	Asteraceae	*Artemisia afra*	African wormwood	Leaves and stem
9.	Asteraceae	*Artemisia vulgaris*	Mugwort	Leaves and stem
10.	Aristolochiaceae	*Asarum canadense*	Wild ginger	Rhizomes
11.	Asteraceae	*Cichorium intybus.*	Chicory	Leaves
12.	Amaryllidaceae	*Crinum moorei*	Inanda lily	Leaves and stem
13.	Cyatheaceae	*Cyathea dealbata*	Tree fern	Leaves and stem
14.	Poaceae	*Cymbopogon nardus*	Lemon grass	Leaves
15.	Apiaceae	*Foeniculum vulgare*	Fennel	Stem and fruit
16.	Lamiaceae	*Mentha piperita*	Peppermint	Leaves and stem
17.	Moringaceae	*Moringa oleifera*	Horse radish tree	Leaves
18.	Lamiaceae	*Ocimum gratissimum*	African basil	Leaves and fruit
19.	Poaceae	*Oxytenanthera abyssinica*	Lowland bamboo	Stem
20.	Lamiaceae	*Plectranthus* sp1	Supr flower	Leaves and bark
21.	Lamiaceae	*Plectranthus* sp2	Supr flower	Leaves and bark
22.	Lamiaceae	*Plectranthus* sp3	Supr flower	Leaves and bark
23.	Lamiaceae	*Plectranthus* sp4	Supr flower	Leaves and bark
24.	Myrtaceae	*Psidium guajava*	Guava	Leaves
25.	Rubiaceae	*Psychotria capensis*	Bird berry	Leaves
26.	Myrtaceae	*Syzygium aromaticum*	Clove	Fruit
27.	Myrtaceae	*Syzygium cordatum*	Water berry	Leaves
28.	Canellaceae	*Warburgia ugandensis*	East African pepper	Leaves and bark
29.	Canellaceae	*Warburgia salutaris*	Pepper bark	Leaves and bark

a private garden in Pietermaritzburg, South Africa. *Allium sativum* bulbs and *Syzygium aromaticum* (clove) were purchased from a local market. All plants used were identified by the Department of Botany, UKZN. Voucher specimens of plants were deposited at the UKZN Herbarium, Pietermaritzburg and were assigned voucher numbers. Plant samples were washed and cut into small pieces, air dried under shade, and then dried in an oven (Model 5SOE1B, Labcon, Maraisburg, South Africa) at 50°C for 3 – 5 days. All plant samples were ground into a fine powder using an electric grinder (Retsch Haan, Germany). Crude powders fitted with a 1 mm diameter sieve, then preserved in airtight plastic containers, labeled and stored at 4°C until used in extract preparation. The same plant samples were used for all subsequent experiments.

Extraction procedure

Plant extraction techniques were carried out in the Department of Animal and Poultry Science, School of Agricultural, Earth, and Environmental Sciences (SAEES), UKZN. Extraction was carried out using ethanol as the solvent, which has been consistently proven to be the most effective solvent for extracting potentially bioactive compounds (McGaw & Eloff, 2008; Ahmed *et al*., 2012). Extraction was performed following (Ahmed *et al*., 2012). Ten grams of each plant sample were boiled in 100 mL of 80 % ethanol for 24 hours using a Soxhlet extractor. Each extract was transferred to a beaker and placed in a water bath (Model 101, Labotec, South Africa) at 60°C. Extracts were evaporated till dry. The dried extracts were stored in sealed glass vials in the dark at 4°C until tested for antihelmintic efficacy. The dried extracts were reconstituted in 80 % ethanol to bring up the concentration required for a specific assay.

Collection of faecal samples

Seventy-two fecal samples from randomly selected cattle from three different areas in Pietermaritzburg, KwaZulu-Natal Province, Impendle, (latitude 29.599696 S, longitude 29.867056 E, 6000 meters above sea level), the Cedara Research Institute (latitude 29.32 S, longitude 30.16 E, 1085 meters above sea level) and Mpumuza, (latitude 29.62 S, longitude 30.38 E, 596 meters above sea level). Twenty-four fecal samples were collected from naturally infected cattle (1 – 4 years old) in each area. All experimental animals were allowed to graze freely on planted Kikuyu

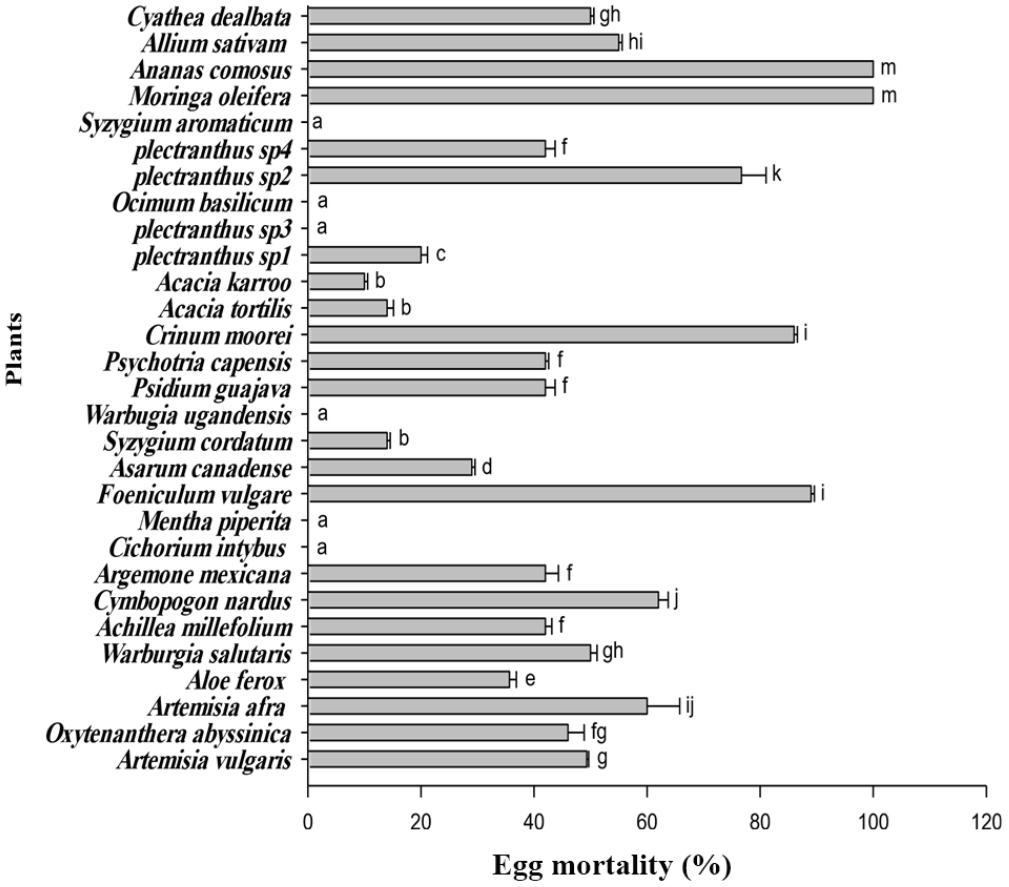

Fig. 1. The effects of ethanolic extracts of 29 plants on the mortality of eggs of liver flukes from cattle, observed for 15 days after treatment. Mortality was zero in the control treatment.

Table 2. ANOVA table for the main effect of 29 ethanolic plant extracts against eggs of liver flukes from cattle.

Source of variation	d.f.	s.s.	m.s.	F value	P value
Plants	29	87998.22	3034.421	320.08	<0.001
Residual	58	549.844	9.48		
Total	89	88556.22			
LSD	5.032				
Mean ± SE	41.56 ± 2.514				
CV%	7.4				

pasture (Pennisetum clandestinum Hochst. ex Chiov.) under the same conditions as other animals. The experimental animals were also treated with an antiparasitic drug, following the deworming protocol used in each farm. Rectal fecal samples were taken by hand and stored in plastic bags. Samples were transported to the Department of Plant Pathology, UKZN, where egg samples were prepared and treated.

Preparation of the liver fluke eggs

For each sample, 100 g of feces was weighed and placed in a 1000 ml beaker filled with 500 ml distilled water. The contents were mixed thoroughly using a glass rod and poured through a tea strainer to remove large debris. The solution was poured into a 1000 ml conical flask, and the suspension was sedimented overnight. The supernatant was then removed. To obtain pure eggs, samples were passed twice through a 100 mm diameter sieve (330 mm pore size).

Egg samples were stored in sterilized sealed bottles at 4°C for further use.

Screening of ethanolic extracts for ovicidal activity against eggs of liver flukes

Ethanolic extracts of 29 plants were evaluated for their ovicidal efficacy against eggs of liver flukes at a 20 % concentration of the raw extract. Treatments were prepared by pipetting 125µl from the egg samples (containing approximately 10 eggs) into 96 wells of cell culture plate. From each plant extract, 125µl was added to the egg samples (3 replicates each). Two controls were undertaken: one treated with ethanol and one with distilled water. To determine the ovicidal activity of each plant extract, the treated eggs were incubated at 28°C for 15 days. Miracidial formation was observed under a light microscope using a 10-magnification.

The experiment was arranged in a randomized complete blocks design (RCBD).

Concentration-response bioassay

The five plant extracts that caused the highest mortality levels in the primary bioassay were selected to evaluate the effect of concentrations on their efficacy. All samples were then used at three concentrations, 5, 10 and 20 % of the raw extract. Each concentration was used to treat fluke egg samples. As in the first part of the experiment, ethanol and distilled water are used as controls. The experiment was a 5 x 3 factorial arranged in a randomized complete block design (RCBD).

Control mortalities were zero in both experiments. Data was subjected to analysis of variance (ANOVA) using GenStat for Windows, 18th edition (Payne *et al*., 2014). Means were compared using Fisher's Least Significant Difference at a 5 % significance level.

Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed.

Table 3. ANOVA table for the activity of five plant extracts at three concentrations against eggs of liver flukes from cattle.

Source of variation	d.f.	s.s.	m.s.	F value	P value
Plants	5	19631.7	3926.34	67.51	<0.001
Concentrations	2	8405.81	4202.91	72.26	<0.001
Plants x concentrations	10	1896.41	189.64	3.26	0.005
Residual	34	1977.52	58.16		
Total	53	31998.59			
LSD	13.51				
Mean ± SE	67.4 ± 6.23				
CV%	12.65				

Table 4. *In vitro* efficacy of ethanolic extracts of five plants against eggs of liver flukes from cattle..

Plants	5% Extract Mortality (%)	10% Extract Mortality (%)	20% Extract Mortality (%)
Moringa oleifera	82.67 fg	90 gf	100 h
Ananas comosus	80 fg	89 gf	100 h
Foeniculum vulgare	63 de	75 ef	89 gf
Cymbopogon nardus	33 a	47 bc	62 de
Artemisia afra	30 a	42 ab	60 cd

Means followed by the same letter do not differ significantly at P < 0.001, according to the Duncan's multiple range test. Mortality was zero in the control treatment.

Results

Effects of ethanolic plant extracts on eggs of liver flukes from cattle
There were highly significant differences in the effects of plant extracts in their effects on eggs of liver flukes of cattle at a 20 % concentration *(F*= 320.08; *P<0.001)* (Table 2). Mortality levels ranged from 0 to 100 % (Fig. 1). Extracts of two plants, *M. oleifera* and *A. comosus*, caused the highest mortality levels of 100 %. Extracts of three plants, *F. vulgare*, *C. moorei* and *Plectranthus* sp2, caused mortality levels higher than 80 %. Extracts of three plants, *C. nardus*, *A. afra* and *W. salutaris*, caused 50 to 62 % mortality levels. Extracts of the remaining 24 plants caused mortality levels of less than 50 % (Fig. 1).

Screening of three concentrations of plant extracts on liver flukes
There were highly significant differences between the plant extracts (*F*=67.51*; P<0.001*) and between the three concentrations (*F=72.26; P<0.001*). The interaction between plant extracts and concentrations was also significant (*F=3.26;* P<0.005) (Table 3). The five selected plant extracts were effective at the three concentrations of 5, 10 and 20 % against the eggs of liver flukes. Mortality levels ranged from 30 – 82.67 % due to the 5 % extracts, 42 – 90 % for the 10 % extracts and 60 – 100 % for the 20 % extracts. Extracts of *M. oleifera* and *A. comosus* caused more significant mortalities than the other plant extracts at the 5, 10 and 20 % concentrations (Table 4). The concentration of 20 % was consistently

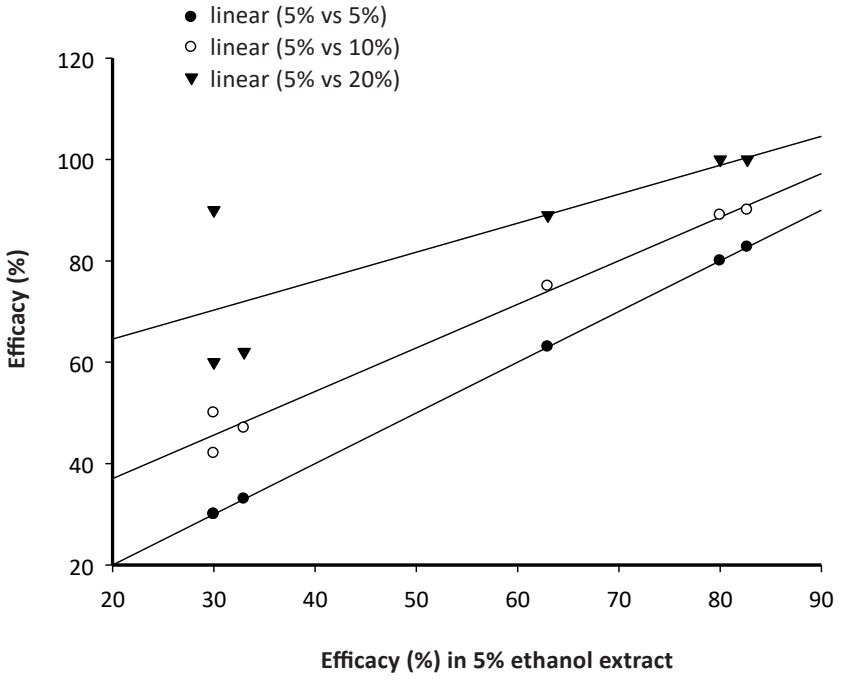

Fig. 2: *In vitro* efficacy of three concentrations of five ethanolic plant extracts against eggs of liver flukes from cattle.
5% versus 10%: y = 13.8x + 27.2 R^2 = 0.9115; 5% versus 20%: y = 11.8x + 46.8 R^2 = 0.8808
▼ – five plants at 20% concentrations; O – five plants at 10% concentrations; ● – five plants and bromelain at 5% concentrations

more effective than 5 or 10 %. A relatively constant performance ranking resulted in straight-line regressions for efficacy x concentrations (Fig. 2).

Discussion

This study evaluated the ovicidal activity of ethanolic extracts of 29 selected plants to inhibit miracidia formation of liver flukes. These plant extracts caused mortality levels of 0 to 100 %. Extracts from five plants (*M. oleifera*, *A. comosus, F. vulgare*, *C. moorei* and *Plectranthus* sp2) strongly inhibited the hatching and development of eggs of liver flukes. Fahey (2005) and Wang *et al*. (2016) reported that *M. oleifera* contained various bioactive compounds with pharmacological activities such as anthelmintic properties. Pereria *et al*. (2016) noted the potential of *Momordica charantia* (L.) leaf-cured extract (CE) and its sub-fractions against the eggs of *F. hepatica*. After 12 days, no miracidia were formed in eggs incubated with *M. charantia* leaf CE at concentrations above 12.5 mg ml^{-1}. The CE sub-fraction, at concentrations of 1000, 100, 10, 0.1 and 0.01g ml^{-1}, affected the development of miracidia, with n-butanol causing the strongest inhibition of miracidia formation. Chemical analysis of the leaf extract suggested that flavonoids were responsible for this effect (Pereria *et al*., 2016). Jeyathilakan *et al*. (2012) evaluated aqueous extracts of *Allium sativum* (L.)*, Lawsonia inermis* (L.) and *Opuntia ficus-indica* ((L.) Mill.) against *F. gigantica* adults at concentrations of 1, 2.5 and 5 % of the raw extract. Extracts of *O. ficus-indica* exhibited flukicidal effects at 2.5 and 5 % concentrations, whereas the other plants were effective at higher concentrations only. A methanolic stem bark extract of *Acacia senegal* (L.) against adults of *F. gigantica* from cattle was evaluated by Alsadeg *et al*. (2015). Anthelmintic activity with mortality levels of 100 % occurred at 1000 mg kg^{-1} and 500 mg kg^{-1} concentrations 6 and 12 hours after exposure, respectively. Ferreira *et al*. (2011) found trematodicidal activity when using the ethanolic extracts of *Artemisia annua* (L.), *A. absinthium* (L.), *Asimina triloba* ((L.) and *Fumaria officinalis* (L.) *in vitro* against mature *Schistosoma mansoni* (Sambon)*, F. hepatica*, and *Echinostoma caproni (*Rudolphi). In the same context, Hossain *et al*. (2013) reported that a methanolic extract of *Dregea volubilis* ((L.) Benth. Ex Hook.), used at a concentration of 100 mg mL^{-1} had flukicidal activity against adults of *F. hepatica*.

Plants with the best activity in the first screening were compared in concentrations of 5, 10 and 20 % in a dose-response assay against the eggs of liver flukes. The highest concentration of 20 % was consistently better than the 5 and 10 % concentrations. However, there was no significant difference in activity between the 5 and 10 % extracts when using *M. oleifera* and *A. comosus*. Extracts of *M. oleifera* and *A. comosus* were more active than the extract of other plants at all concentrations. These results are in line with the findings of Moazeni & Khademolhoseini (2016), who studied the ovicidal activities of methanolic extracts of *Zingiber officinale* (L.) against eggs of *F. hepatica* at concentrations of 1, 5, 10, 25 and 50 mg ml^{-1}. Control levels of 100 % were observed even at lower concentrations of 5 and 10 mg ml^{-1} after 48 and 24 hours of exposure time, respectively. Moxonet *et al*. (2010) reported that many proteins expressed during the early stages of embryogenesis are directly involved in cellular proliferation and cytoskeleton organization events. Thus, it is possible that compounds such as alkaloids, flavonoids and tannins present in *M. oleifera*, and bromelain in *A. comosus*, interact with the protein expression profile and therefore inhibit the development of eggs. However, further research is needed to conduct toxicity tests and determine how each plant extract affects protein expression and fluke egg development.

Conclusions

Ethanolic extracts of *M. oleifera* and *A. comosus* can inhibit the hatching and development of liver fluke eggs from cattle *in vitro,* even at low concentrations. Regularly adding these plants or plant extracts into animal feed could reduce the incidences of fascioliasis.

Conflicts of Interest

The authors have no potential conflict of interest pertaining to this submission to Helminthologia.

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