ORIGINAL ARTICLE

DIATOMACEOUS EARTH INHIBITED THE *IN VITRO* MIGRATION OF *Oesophagustomum dendatum* LARVAE

Ma. Asuncion G. Beltran[1] and Richard Martin[2]

An alternative anthelmintic diatomaceous earth (DE) was evaluated for its ability to inhibit the migration of *Oesaphagostomum dentatum* larvae using migration and inhibition assays *in vitro* in unsheathed and exsheathed third stage larvae. The experiments were tested in 24-well plates at room temperature with five replications per treatment using different DE concentrations of 0.1 mg/ml, 0.3 mg/ml and 1mg/ml. About 120 larvae per well were deposited on a larval migration apparatus consisting of 20 µm nylon mesh filters and incubated in 2, 4, 16, 20 and 24 hr under different treatments and another 2 hr to allow the migration of active motile larvae. The percentage inhibition were statistically analyzed. The highest inhibition was 78.20%, observed when DE was given at a dose of 0.3 mg/ml after 24 hr (P<0.001) for exsheathed larvae and 67.60% (P<0.01) with unsheathed larvae under 1 mg/ml concentration of DE after 24 hr. DE was more effective in exsheathed larvae at 0.3 mg/ml after 20 hr with 61.60% inhibition (P<0.01). With unsheathed larvae, DE had a significant effect at 1 mg/ml exposed within 24 hr with 67.60% inhibition (P<0.01). The larval migration/inhibition assay presented in this study showed the inhibitory effects of DE on *O. dentatum*.

Keywords: anthelmintic, diatomaceous earth, larval migration assay, *Oesophagostomum dentatum*

INTRODUCTION

Diatomaceous earth (DE), or diatomite, is the fossilized remains of diatom shells. After quarrying, crushing and milling, a fine light dust is obtained with certain abrasive properties and the ability to absorb lipids to about three times or more of its particle mass (Korunic, 1998). DE with less than 7% composition of crystalline silica is generally recognized as a safe feed additive in Canada and the U.S., and is approved as a feed supplement by organizations such as the FDA and USDA (Bennett *et al.,* 2011, Köster, 2013).

DE is often touted as an effective and alternative anthelmintic for sheep, goats, cattle, poultry and other livestock, and is classed as a feed ingredient for livestock health care. DE is used to control many invertebrate pests, including grain storage invertebrates, and for gastro-intestinal parasite control (OMRI, 1997), although the small number of efficacy studies show mixed results (Fernandez *et al.*, 1998; McLean *et al.,* 2005; Bennett *et al.,* 2011). It is hypothesized that DE kills worms by slashing them with its rigid, blade-like surfaces. The silaceous material damages the invertebrate cuticle (arthropods and nematodes), which increases permeability and then causes death by dehydration (McLean *et al.,* 2005). However, there is a lack of scientific evidence

[1]Tarlac College of Agriculture, Camiling, Tarlac, Philippines (email: marizonbeltran@yahoo.com), [2]Iowa State University, Iowa, U.S.A.

ABSTRACT

to support its use (Schoenian, 2013). To expand recent knowledge, DE was tested to evaluate its effect on *Oesophagostomum dentatum* larvae. *Oesophagostomum* is a genus of parasitic roundworms belonging to the family called *Strongylidae* that affect cattle, sheep, goats and other ruminants, as well as pigs and wildlife (deer, antelope, camels, monkeys, wild boars, etc.). It is found worldwide, but is more frequent in warm and humid climates in tropical and subtropical regions (Junquera, 2014).

This study tested the anthelmintic effects of diatomaceous earth on *O. dentatum* larvae using the larval migration and inhibition assay *in vitro.* Larvae were used because of their availability and to potentially serve as a model for future studies.

MATERIALS AND METHODS

Diatomaceous earth

Food grade diatomaceous earth (100%, uncalcined) was purchased from a U.S. supplier (St. Gabriel Organics®, Orange, Virginia). It contains >85 % SiO_2 (silica, amorphous), <0.1% crystalline silica, <10% other elemental oxides, and ~5% moisture (Welborn, 2011). It is an off-white powder, light in weight and chalk-like in appearance. It has a particle size ranging from less than 1µ to 1ml, but typically 10 to 200 µ (Koster, 2013). The different treatments were prepared in 0.1 mg/ml, 0.3 mg/ml and 1.0 mg/ml concentrations of diatomaceous earth.

Drug treatments

Two studies were carried out to assess the effect of diatomaceous earth on a) unsheathed, and, b) exsheathed larvae of *O. dentatum. In vitro* tests were carried out in both studies. There were six treatments, being: one negative control (migration buffer), two positive controls (levamisole and albendazole) and DE 0.1mg/ml, 0.3 mg/ml and 1 mg/ml concentrations. Levamisole and albendazole (Fisher® Scientific, Pittsburgh, Pennsylvania), were both used at 30 µM concentrations. Levamisole and DE were suspended in the migration buffer while albendazole was first dissolved in DMSO and then suspended in migration buffer. All treatments were at 7.0 pH. The experiments were carried out in 24-well plates at room temperature (20-25^{o}C) with five replications per treatment.

Oesophagostomum dentatum larvae

Oesophagostomum dentatum larvae were cultured from faecal matter of pigs, collected in a piggery farm near Iowa State University, Ames, Iowa, USA. It was mixed daily with wooden spoons and incubated for 6 days to promote egg hatching and the development of infective larvae. The L3 larvae were recovered by the Baerman funnel technique and were filtered and washed by spinning down with 40% sucrose. Afterwards, the larvae were re-suspended in sterile distilled water and stored in culture flasks and maintained in a Low Temperature Incubator™ (Fisher® Scientific, Pittsburgh, Pennsylvania) at 10.5^{o}C. For ex-sheathment, the larvae were placed in a Falcon tube with 5 ml of sterile water and one ml of 10% of sodium hypochlorite (Vertex CSS-5 Bleach®, St. Louis, MO) for 5-8 min at room temperature, or, as soon as 90-95% of the larvae were exsheathed when seen under a microscope (Nikon TMS™, MVI, Avon, Maine). The mixture then was spun (Eppendorf Centrifuge 5415, Brinkmann™ Instruments, Inc. Westbury, New York) at 31,000 rpm for 2 min, rinsed and re-suspended in sterile water**.**

Anthelmintic activity: larval migration assay

To perform the larval migration assay, the procedure used by Nagamori *et al.*

(2013) was used. Unsheathed larvae of *O. dentatum* were suspended in the different concentrations of diatomaceous earth in 1.5 ml micro tubes (Fisher® Scientific, Pittsburgh, Pennsylvania). The concentrations of the larvae were at 120 larvae/well. In the other experiment, larvae were then exsheathed using a 10% bleach (hypochlorite) solution, followed by washing with migration buffer. After washing, they were re-suspended in appropriate positive and negative control drugs and each of the above indicated concentrations of DE (St. Gabriel Organics®) and incubated at room temperature (25-30°C). Observations for each experimental unit were taken at 2, 4, 16, 20 and 24 hr time intervals.

At the end of each incubation period, the larvae were added to the migration apparatus consisting of 20 µm nylon mesh filters (Elko®) in a 24-well plate (Corning®, Corning, New York) and incubated for 2 hr to allow migration/inhibition of the L3 active, motile larvae. After the 2 hr migration periods, the larvae were washed with migration buffer into 3.5 cm petridishes (Fisher Scientific®). Both the number of migrated larvae from the 24-well plate and inhibited larvae from the dishes were recorded under a microscope (Nikon TMS™, MVI, Avon, Maine), as well as the total number of larvae. The evaluation criteria were based on the average number of inhibited larvae under the different treatments. The percentage inhibition was calculated by taking the number of inhibited larvae divided by the total number of larvae and multiplied by 100.

Statistical analysis

Data were statistically analyzed using two-way analysis of variance (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, California). The differences between the negative control and each of the treatments were analyzed using the BonFerroni post-test using the same program.

RESULTS AND DISCUSSION

Table 1. Mean percentage of inhibition of diatomaceous earth (DE) on unsheathed larvae of *Oesophagustomum dentatum*.

Treatment	% Mean Inhibition+				
	2 hr	4 hr	16 hr	20 hr	24 hr
Migration Buffer (-) Control	3.80 ns	16.80 ns	24.80 ns	30.20 ns	40.00 ns
Levamisole (+) Control	45.00***	66.00***	76.40***	93.40***	93.80***
Albendazole (+) Control	36.40***	63.80***	81.60***	87.20***	92.80***
DE 0.1mg/ml	17.00ns	27.80 ns	37.80 ns	39.60 ns	46.80 ns
DE 0.3 mg/ml	21.20 ns	28.80 ns	37.20 ns	46.20 ns	53.40 ns
DE 1.0 mg/ml	21.80 ns	31.60 ns	39.40 ns	52.40*	67.60**

+ Bonferroni Post-tests results as compared to the negative control (P value= <0.01).
ns no significant differences
* Significant result at P<0.01
** Highly significant result at P<0.001
*** Highly significant result at P<0.0001

Table 1 shows the effect of DE on unsheathed larvae of *O. dentatum.* There were highly significant differences among the treatment groups (P<0.0001). Variation in the % inhibition by dosage rates and times of exposure to DE also proved to be very significant (P<0.0001). The dosage rates of DE accounted for 52.16% (P<0.0001) of the total variation, while 29.20% (P<0.0001) of the total variation was accounted for by the times of exposure, although the interaction was not significant at 2.88% (P=0.3640). DE exhibited the highest inhibition at 52.40% when treated at the highest dose of 1 mg/ml and exposed about 20 hr (P<0.05), and had 67.60% inhibition after 24 hr of exposure (P<0.01) in unsheathed larvae. Levamisole and albendazole (chemical dewormers), being the positive controls, delivered highly significant effects on the inhibition of the larvae (P<0.0001), as expected.

Effects of DE on exsheathed larvae of *O. dentatum* are shown in Table 2. The results show highly significant differences among the treatment groups (P<0.0001). Variations in the % inhibition by dosage rates and times of exposure to DE also proved to be highly significant (P<0.0001). The dosage rates of DE accounted 45.76% (P<0.0001)

Table *2*. Mean percentage of inhibition of diatomaceous earth (DE) on exsheathed larvae of *Oesophagustomum dentatum.*

Treatment	% Mean Inhibition+				
	2 hr	4 hr	16 hr	20 hr	24 hr
Migration Buffer (-) Control	9.40 ns	30.80 ns	35.00 ns	40.40 ns	50.00 ns
Levamisole (+) Control	65.80***	71.40***	89.00***	97.20***	97.40***
Albendazole (+) Control	42.00***	64.60***	93.40***	98.60***	99.40***
DE 0.1mg/ml	15.80 ns	26.80 ns	39.60 ns	53.40ns	63.00 ns
DE 0.3 mg/ml	22.00 ns	39.40 ns	44.80 ns	61.60**	78.20***
DE 1.0 mg/ml	21.40 ns	43.40 ns	54.00*	63.40**	78.20***

+ Bonferroni Post-tests results as compared to the negative control (P value= <0.01).
ns no significant differences
* Significant result at P<0.01
** Highly significant result at P<0.001
*** Highly significant result at P<0.0001

of the total variation while the times of exposure accounted for 40.25% (P<0.0001) of the total variation. The interaction accounted for 4.33% of the total variance (P=0.0005) which was also highly significant.

DE had the highest inhibition when *O. dentatum* larvae were exsheathed compared with the unsheathed larvae: 61.60% inhibition was observed when DE was given at a dose of 0.3 mg/ml with time of exposure of about 20 hr (P<0.01) and a 78.20% inhibition after 24 hr (P<0.001) of exposure to DE. When DE was given the highest dose of 1 mg/ml and larvae were exposed for at least 16 hr, 54.00% inhibition was observed as early as 16 hr (P<0.001) and progressed to 78.20% after 24 hr of larval exposure.

Since there is a widespread interest in using DE as a natural anthelmintic, this study documented that DE has a positive effect on inhibition of O. *dentatum* larvae *in vitro*. The results indicate very significant effects on the percentage inhibition, particularly when DE was applied at the highest dosage rate (1mg/ml) and the larvae were exposed

for 20 hr or more for exsheathed larvae, and with 0.3 mg/ml at 20 hr exposure with unsheathed larvae. The DE mode of action that immobilizes the larvae is unclear, but it has been suggested that the abrasive action of the powder pierces or scratches the outer protective layer of invertebrates, including internal parasites, resulting in death by means of dehydration (McClean, 2005). When infective larvae were immobilized, there was an inhibition of energy production that resulted in decreased protein synthesis (Van des Borsche, 2012). Furthermore, ATP production is inhibited and the effects may result in the formation of actomyosin with consequent muscle stiffness in parasitic larvae (Van des Borsche, 2012). With this mode of action where the larvae are immobilized and eventually die, the capacity of the parasitic larvae to infect animals will be reduced.

In contrast, in *in vivo* studies on DE conducted by Bernard *et al.* (2009) where DE was used in infected goats, there was no significant reduction in fecal egg count and the anthelmintic effect of DE was not observed. Similar results were found in sheep (Osweiler and Carson, 1997). The results of their studies did not indicate significant effects of DE on the parasite load as measured by eggs per gram of feces, and DE did not reduce anaemia as measured by packed cell volume and other blood parameters (Schoenian, 2013). However, this study is the first to report positive effects of DE against parasitic larvae done *in vitro.*

CONCLUSION

If the practical minimum acceptable level of inhibition of the larvae or a benchmark level of a natural product is for example 60%, the result of using DE was significant. DE was more effective in exsheathed larvae at 0.3 mg/ml and exposed at 20 hr and delivered 61.60% inhibition effect ($P<0.01$). While with unsheathed larvae, DE has significant effect when given at 1 mg/ml exposed within 24 hr with 67.60% inhibition effect ($P<0.01$). The larval migration/inhibition assay presented in this study may provide a methodology to obtain measures of the inhibitory effects for DE and other natural products on other parasites in the future. Further studies may be conducted on different larvae species, and field experiments may be conducted under varied pasture management techniques.

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