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The Effects of Plant Leaves Variants from The Philippines on Infective *Oesophagustonum dentatum* Larvae

Pengaruh Sembilan Tanaman Asal Filipina Terhadap Infeksi Larva Oesophagustonum dentatum

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ABSTRACT

Nine aqueous extracts of plant leaves from the Philippines were evaluated against *Oesaphagustomum dentatum* sensitive (ODSEns) larvae for their anthelmintic effects. The plant species evaluated were *Ananas comosus, Chrysophyllum cainito,Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl,*Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala*. The leaves of the plants gathered from Camiling, Tarlac, Philippines, were dried under room temperature for 30 days and then ground. Aqueous extracts of these plant leaves were prepared using migration buffer. *In vitro* migration and inhibition assays were used to evaluate the anthelmintic activity against exsheathed third stage larvae of *Oesophagustomum dentatum.* The experiments were put in 24-well plates at room temperature with four replications per treatment using different concentrations of 1, 2.5, 5 and 10 mg/ml. About 120 larvae per well were deposited on a larval migration apparatus consisting of 20 µm nylon mesh filters and incubated 20 hours under different treatments and another 2 hours to allow the migration of active motile larvae. The evaluation criteria were based on the average numbers of inhibited larvae under the different treatments. The percentage inhibition were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The most effective leaves with their highest inhibition level were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (\Makabuhay, 55.75%), and *Azadirachta indica* (Neem, 51.50%), whereby extremely significant (P<0.001) effect against *O. dentatum* larvae. Two leaves extracts, from *Trichantera gigantea* and *Leuacaena leucocephala* have no significant effect on *O. dentatum* larvae. The larval migration assay allows initial studies on the anthelmintic effects of the herbs.

Keywords: plant leaves, anthelmintic property, inhibition assay, Philippines

ABSTRAK

Sembilan ekstrak air daun tanaman dari Filipina dievaluasi terhadap sensitifitas larva *Oesaphagustomum dentatum*yang mempengaruhi anthelmintik. Spesies tanaman yang dievaluasi adalah *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii Boerl, Azadirachta indica, Trichantera gigantea* dan *Leuacaena leucocephala*. Daun tanaman dikumpulkan dari Camiling, Tarlac, Filipina, dikeringkan selama 30 hari dalam suhu kamar dan kemudian ditumbuk. Ekstrak air dari daun tanaman tersebut disiapkan menggunakan buffer migrasi. Migrasi in vitro dan uji penghambatan dilakukan untuk mengetahui aktivitas antelmintik terhadap larva tahap ketiga *Oesophagustomum dentatum.* Eksperimen dilakukan dalam 24-sumuran *plate* pada suhu kamar dengan empat replikasi per perlakuan menggunakan konsentrasi yang berbeda dari 1, 2,5-, 5 dan 10 mg / ml. Sekitar 120 larva per sumur diendapkan pada peralatan migrasi larva yang terdiri dari 20 µm filter mesh nilon dan diinkubasi 20 jam dengan perlakuan berbeda dan 2 jam lagi untuk memungkinkan

migrasi larva motil aktif. Kriteria evaluasi didasarkan pada jumlah rata-rata larva yang dihambat di bawah perlakuan yang berbeda. Penghambatan persentase dianalisis secara statistik menggunakan Two-Way ANOVA (P = ≤,0001) menggunakan *software* Graph Pad Prizm (V5 San Diego, Ca). Daun yang paling efektif dengan tingkat penghambatan tertinggi berasal dari *Ananas comosus* (nanas, 86,50%), *Gliricidia sepium* (daun Gamal, 84,50%), *Chrysophyllum cainito* (daun sawo duren, 83,75%), *Moringa oleifera* (daun kelor, 58,75%), *Annona squamosa* (daun srikaya, 56,00%), *Tinospora rumphii Boerl*(Bratawali, 55,75%), dan *Azadirachta indica* (daun mimba, 51,50%), semuanya menunjukkan efek yang sangat signifikan (P<0,001) terhadap larva *O. dentatum*. Dua ekstrak daun yaitu *Trichantera gigantea*(daun cemara raja) dan *Leuacaena leucocephala*(daun lamtoro) tidak berpengaruh signifikan terhadap larva *O. dentatum*. Uji migrasi larva memungkinkan studi awal tentang efek anthelmintik dari herbal**.**

Kata kunci**:** daun tanaman, antihelmintik, uji penghambatan, Filipina

INTRODUCTION

The high cost of conventional anthelmintic drugs and the development of anthelmintic resistance lead researchers to evaluate the different plants as an alternative source of anthelmintics, especially in tropical countries where many plants abound throughout the year. The use of plants and plant extracts as a possible source of natural anthelmintics has received more interest in the present years.

The use of medicinal plants for the prevention and treatment of gastro-intestinal parasitism has its origin in ethnoveterinary medicine (Athanasiadou *et al*, 2007). For ages, plants have been used to control parasitism, and in many countries of the world, they are still used for this purpose. There have been reports around the world about the use of plants for their anthelmintic properties to treat human and animal parasitic infection (Bahaud, *et al*, 2006; Lopez-Aroche *et al*, 2008; Mali and Mehta, 2008; Bachaya *et al*, 2009; Marie-Magdeleine, *et al*, 2010; Narojini *et al*, 2011; Rabiu and Subhasish, 2011; Reuben *et al*, 2011; Deb Roy *et al*, 2012; Getachew *et al*, 2012 and Ferreira *et al*, 2013). The aim of this study was to test anthelmintic effects of nine plants aqueous extracts from the Philippines under controlled experimental conditions.

MATERIAL AND METHO DS

Plant leaves

Plants leaves were collected fresh, directly from naturally grown plants in Camiling, Tarlac, Philippines during the wet season (July and August). Leaves were dried for 30 days under room temperature and then sent to Iowa State University with an approved permit. The plant leaves were taxonomically identified as *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl, *Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala.* The scientific names and common names are summarized in **Table 1**.

Plant extract preparations

The leaves were ground using an Oster® processor at high speed for two minutes and stored in 50 ml falcon tubes until later use. One gram of leaves of each species were suspended in 20 ml of migration buffer and were shaken for 24 hours using an Orbit1000® (Labnet Intl). The aqueous solution was then extracted using 20 μm nylon mesh (Elko®) filters thus making the stock solution in equivalent to 0.05 g/ml concentration. Each treatment was prepared adding 0.2 ml, 0.5 ml, 1ml and 2 ml and up to 10 ml of the migration buffer. The

concentrations were prepared at 1 mg, 2.5 mg, 5 mg and 10 mg/ml, respectively.

Oesophagustomum dentatum L_3

The *Oesophagustomum dentatum* larvae were obtained by fecal culture. The feces were mixed daily with wooden spatulas and incubated for 6 days to promote egg hatching and the development of L_3 infective larvae. The L larvae were recovered using the Baerman funnel technique, and filtered and washed by centrifuging in 40% sucrose.Afterwards, they were re-suspended in sterile distilled water and stored in culture flasks and maintained in a low temperature Incubator™ (Fisher Scientific) at 10.5^{o}C.

For exsheathment, the larvae were placed in sterile water in a5 ml Falcon® tube with one ml of 10% of sodium hypochlorite (Vertex CSS-5 Bleach®, St. Louis, MO) for 5-8 minutes at room temperature or as soon as 90-95% of the larvae were exsheathed when seen under a microscope (Nikon TMS™, MVI, Avon, Ma.). The mixture was spun (Eppendorf Centrifuge 5415, Brinkmann™) at 31,000 rpm for 2 minutes, rinsed and re-suspended in sterile water.

Anthelmintic Activity: Larval migration assay

For the larval migration assay, the procedure of Nagamori, *et al* (2013) was used. The concentrations of the larvae were at 120 larvae/well. After the larvae were exsheathed, they were re-suspended in appropriate positive and negative control drugs and with the different leaves extracts in aqueous solution at concentrations of 1mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml, and then incubated at room temperature (25-30^{O}C) for 20 hour treatment exposure time periods.

After twenty hours, the larvae were added to the migration apparatus consisting of 20 µm nylon mesh filters (Elko®) in a 24-well plate (Corning®, Corning, NY) and incubated for another 2 hours to allow migration/inhibition of the L3 active, motile larvae. After 2 hour migration periods, the larvae were washed with migration buffer into 3.5 cm petridishes . 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, MA), 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 formula was:

$$\% \text{ inhibited} = \frac{\text{No. of inhibited larvae}}{\text{Total No. of Larvae}} \times 100$$

Statistical analysis

Data were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The differences between the negative control and each of the treatments were analyzed using the Bonferroni post-test.

RESULTS AND DISCUSSION

Table 1 shows the mean percentage inhibition to various concentrations of nine different species aqueous leaves extracts after 20 hours of exposure to *O. dentatum* exsheathed larvae. At the highest leaf extracts concentration of 10mg/ml, *Ananas comosus* (pineapple)leaves produced the highest inhibition of 86.50% (P<0.0001), compared to *Gliricidia sepium* (gliciridia) at 84.50% and *Chrysophyllum cainito* (star apple)*at* 83.75% inhibition (P<0.0001). At the same level of concentration, three other plant extracts, *Annona squamosa*had 56.00% (P<0.001) inhibited larvae in the migration assay,

Moringa oleifera with 58.75% (P<0.001) and *Tinospora rumphii* Boerl with 55.75% (P<0.001).

Ananas comosus and *Chrysophyllum cainito* showed very significant effects even when given at a lower concentration of 1 mg/ml, with 49.25% (P<0.001) and 47.75 % (P<0.001) inhibition, respectively (Figure 1). With the study done by Fernandez *et al* (2013), *Chrysophyllum cainito* extract was mixed with other two leaves extracts, makabuhay (*Tinospora rumphii*), and makahiya (*Mimosa pudica*). Ethanol was the best solvent to extract the bioactive components of the three plants. Eighty percent (80%) of *Haemonchus contortus* larvae died 30 minutes after exposure with the cocktail.Likewise, in a number of tests, tannins were reported in the phytochemical analysis of *C. cainito* as being responsible for its observed anthelmintic effects (Fernandez *et al*, 2013). The beneficial effects of condensed tannins on livestock are the reduction of the detrimental effects of parasitism in grazing livestock (Hoste, *et al*, 2006). The consumption of high concentration of condensed tannins (>7% of DM) has been associated with detrimental effects of ruminants such as reduction of food intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen. Low and moderate concentrations of condensed tannins (<6% of DM) have resulted in positive effects in the herbivores like increased growth and increased milk production (Min, *et al*, 2003; Waghorn and McNabb, 2003).

Pineapple leaves have previously been used in the Philippines as anthelmintic preparations for livestock (Jovellanos, 1997; Baldo, 2001). According to Githiori (2004) and Debnath *et al* (2013) the pineapple plant contains cysteine proteases (bromelain), have been reported to have anthelmintic properties.

Table 1. List of medicinal plants used *in vitro* for anthelmintic evaluation against *O. dentatum:* their common name and scientific names

Scientific Name	Common Name
Ananas comosus	Pineapple (Engl); Pina (Spanish); Abacaxi (Portuguese); Annachi pazham (Tamil); Nanas (Malay); Aananas (Many languages)
Chrysophyllum cainito	Caimito (Tag., Span.), Cainito (Engl.) Star apple, Star plum (Engl.) Niu nai guo (Chin.)
Gliricidia sepium	Madre de Cacao (Guatemala) Kakawate (Tag), Cacao de nance, Cacahnanance, Mata Raton, Madriado (Honduras)
Annona squamosa	Custard apple, sugar apple, sweet sop (English); Gishta (Arabic); Kannelappel (dutch), Atis (Tag); Attier, Cachiman canelle, pomme de cannelle (French); Rahmapfel, Süßsack (German); Ata, Sharifa, Sitaphal (Hindi); Buah nona, Nona sri kaya, Sri kaya (Malaysian); Fan-li-chi (Mandarin); Fructa do conde (Portuguese); Anón, Anona, Anona blanca, Candongo, Chirimoya, fructo do conde, pinha, saramuya (Spanish); Lanang, Makkhiap, Noina (Thai); Mang câú ta, na (Vietnamese)
Moringa oleifera	Horseradish tree, Radish tree, Drumstick tree, Mother's Best Friend, West Indian ben (Engl); Bèn ailé, Benzolive, Ben oléifère, Arbre radis du cheval (French); Behenbaum (german); Sàndalo ceruleo (Italian); Acácia branca, Marungo (Portuguese); Árbol del ben, Ben, Morango, Moringa (Spanish); Kachang Kelur (Malasia, Indon); Mulanggay (Tag); Kaanaeng-doeng (Thai)
Tinospora rumphii Boerl	Heavenly elixir (Engl) Makabuhay (Tag., Ilk.); Giloya (Ind.); Fa leng teng (Chin.)
Azadirachta indica	Neem, Nimtree, Indian Lilac, Margosa Tree, Neem Chal (Engl)
Trichantera gigantea	Madre de Agua (Tag), Cenicero, Tuno, Naranjillo, and Palo de agua (Span)
Leuacaena leucocephala	Ipil-ipil (Tag) White Leadtree, White Popinac (Engl) ; Subabool (Indian)

Gliricidia sepium (madre de cacao) was also active with 41.25% inhibition when given at 1mg/ml (P<0.01). Its effect became very significant when exposed at a higher concentration of 5mg/ml, which showed 72.75% inhibition (P<0.001). This validates the efficacy of the leaves extract as it has been used in the Philippines to treat external parasitism. Fresh, clean leaves are pounded, with its juice extracted and applied to affected animal body parts, once to twice a day for one week. Fresh leaves are also used as a fresh feeds for deworming purposes, especially in goats by many farmers in the countryside.

Gliciridia was found to contain 40.7g of condensed tannins/kg dry matter.Tannins have been found to have potential antidiarrheic, antidysenteric, antimutagenic, antinephritic, antioxidant, antiradicular, antiviral, bactericide, cancer-preventive, hepatoprotective, pesticide, psychotropic, and viricide activities (Duke *et al,* 1994). There is little evidence of toxic effects with ruminants fed either fresh or wilted leaves (Stewart, *et al,* 1996). In a reportpublished by the Cornell University (2009), goats safely consumed larger quantities of plants with tannins than cattle and sheep because goats have a salivary protein that binds to the tannins that the other livestock lack. It is reported in Asia that *Gliciridia* has apparent palatability problems (Trung, 1989), but farmers are continuously feeding it, especially in goats, because of its availability. One advantage of *Gliricidia* is having more biomass than *L. leucocephala* (Stewart *et al.* 1992), with annual leaf dry matter production generally ranging from about 2 t/ha/year (Wong and Sharudin, 1986) to 20 t/ha/year (Sriskandarajah, 1987).

Moringa oleifera leaves extract showed 58.75% inhibition, a very significant effect (P<0.001) when given the highest dose of 10mg/ml, and a significant effect of 41.00% inhibition at 5mg/ml concentration (P<0.05). Moringa had been used for a wide range of various ailments (Sreelatha and Padma, 2009). The different parts of this plant such as the leaves, seed, roots, bark, flowers and fruits and immature pods have been found to act as agents for the following effects: cardiac and circulatory stimulus, antiulcer, antispasmodic, diuretic, antihypertensive, possess antitumor, antipyretic, antiepileptic, antiinflammatory, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and treatments of other various ailments in South Asia (Anwar *et al,* 2007). Although, there have been no report on its deworming activity, results of this study found that *Moringa* has potential in this regard.

Annona squamosa leaves extract showed 56.00% inhibition at the highest concentration of 10mg/ml and 48.00% at 5mg/ml (P<0.001) against *O. dentatum* larvae. This conforms with other research where *Annona squamosa*was found to reduce faecal egg counts (FEC) and total worm counts in experimental lambs (Githiori *et al,* 2004).The anthelmintic properties of *Annona squamosa* indicated the presence of phenolic compounds in its aqueous leaf extract (Kamaraj and Rahuman, 2011), which was observed also by Fereira *et al* (2013) when they tested the leaves extract against eggs, infective larvae and adult forms of *Haemonchus contortus* in sheep. Plant phenolic compounds are diverse forms of proanthocyanidins and condensed tannins. They were found to prevent bloat in cattle, reduce gastrointestinal nematode numbers and flystrike (Waghorn and McNabb, 2003).

Tinosphora rumphii or makabuhay leaves (*Tag*.) showed 55.75% inhibition at 10mg/ml concentration (P<0.001), an extremely significant effect, but no

significant effectsat lower concentrations against *O. dentatum* larvae. Earlier studies on crude extracts of *Tinosphora rumphii* were highly effective against *Haemonchus* larvae *in vitro* and in reducing worm egg counts and worm numbers in sheep and goats (Sani *et al*, 2004).

Likewise, *Azadiractha indica* or neem tree leaves produced 51.50% inhibition at 10mg/ml (P<0.01), a very significant effect, but similar to *T. rumphii*, no significant effect was seen when applied at lower concentrations. In tropical Asia, fresh neem tree leaves were fed to sheep, which lowered the infection against *H. contortus* fecalysis count (Sani, 2004). Its active compounds were found to be a high content of condensed tannins, which might be responsible for its anthelmintic activity (Chandrawathani *et al*, 2002 and 2006; Githori *et al*, 2004; Costa *et al*, 2006). **Table 2** presents a summary of several plants used in this study as to their naturally occurring agents that possess anthelmintic properties.

Table 2. Aqueous leaves extracts evaluated against *E. dentatum*, and theirmean percentages of inhibition at different concentrations after 20 hours incubation time under room temperature.

Treatments	% Mean Inhibition* at different concentrations			
	1 mg/ml	2.5 mg/ml	5 mg/ml	10 mg/ml
Migration Buffer (-) Control	18.50	23.75	24.50	29.75
Levamisole (+) Control	94.97	96.00	95.50	98.75
Ananas comosus	49.25***	64.00***	84.50***	86.50***
Gliricidia sepium	41.25**	45.25**	72.75***	84.50***
Chrysophyllum cainito	47.75***	52.50***	59.75***	83.75***
Moringa oleifera	28.75 ns	35.25 ns	41.00 *	58.75***
Annona squamosa	19.75 ns	21.00 ns	48.00***	56.00***
Tinospora rumphii Boerl	11.50 ns	25.75 ns	34.00 ns	55.75***
Azadirachta indica	17.00 ns	22.00 ns	35.00 ns	51.50**
Trichantera gigantea	28.50 ns	31.75 ns	37.25 ns	42.25 ns
Leuacaena leucocephala	5.00 ns	12.50 ns	18.00 ns	21.75 ns

* Bonferroni Post tests results as compared to the negative control (P value= <0.01).
** Significant result at P<0.001.
*** Highly significant results at P<0.0001.

In the data analyses, the interaction between the leaves extracts and the concentrations used account for 5.11% of the total variance (P<0.0001) and was extremely significant. The plant leaves extracts account for 71.41% of the total variance (P<0.0001) and the concentrations account for 15.58% (P<0.0001), both effects were also extremely significant. In summary, the most effective plant species leaves in producing the highest *O. dentatum* larvae inhibition levels were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (Star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (Makabuhay, 55.75%),and *Azadirachta indica* (Neem, 51.50%), which all proved extremely significant (P<0.001). Two plant species leaves extracts, those from *Trichantera gigantea* and *Leuacaena leucocephala*,had no significant anthelmintic effects on *O. dentatum* larvae.

The active compounds are plant secondary metabolites (PSM) (Githiori et al, 2006), the plant products that have been associated with defensive mechanisms of plants against herbivore grazing (Mueller-Harvey and McAllan, 1992). Saponins, alkaloids, non-protein amino acids, tannins and other polyphenols, lignins, glycosides, are all

PSM and some of them have been considered responsible for the anti-parasitic effect of plants (Githiori, 2006).

Fresh roughages supplied to a 30 kgs goat,with 3% of its body weight dry matter (DM) requirement, needs 0.9 kg DM a day. If the roughage to legumes ratio was 75%:25%, the DM needed for roughages was 0.67 kg and 0.22 kg for legumes. Converted the DM into fresh roughage (20% DM), a goat needs about 3.37 kg of roughage and 0.90 kg fresh legumes (25% DM). The legume part was the replaced components by the different leaves in controlling the parasites. Given 10 mg of leaves (DM)/ml as the effective concentration with the highest inhibition effect to larvae, in a goat's rumen with 3 L capacity, at least 151 grams of fresh leaves must be given daily for three consecutive days to effectively control the parasites.

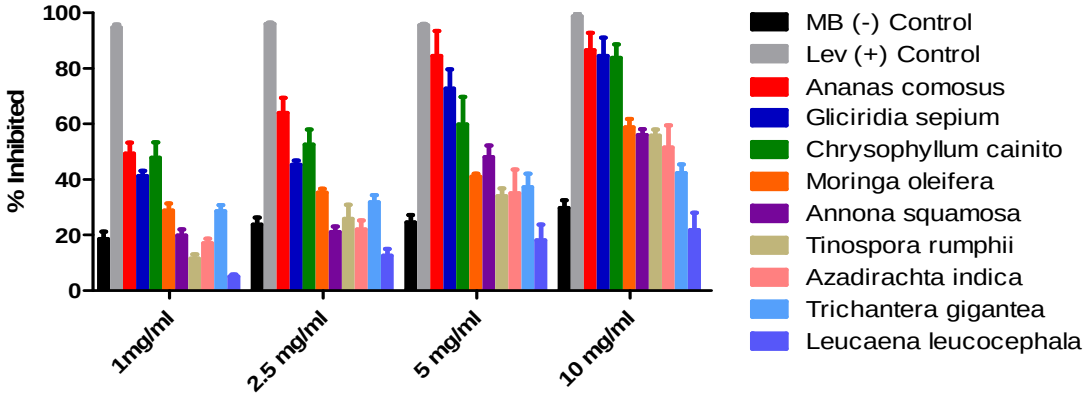

Figure 1. Inhibition percentage of nine aqueous leaves extract on *Oesophagostomum dentatum* larvae using larval migration assay.

CONCLUSIONS

This effort to scientifically screen and evaluate the effect of medicinal plants from the Philippines documented statistically significant inhibition of *O. dentatum* larvae by exposure to the leaf extracts of seven of the nine plant species examined. The results of this study validate the traditional use of these plants as having natural anthelmintic properties and they may be used with its pharmacological potentials for veterinary health management systems, especially in developing countries where these plants abound and the farmers typically lack the financial resources to routinely purchase commercial chemical treatments. The methods of this experiment also provide an additional resource to conduct future *in vivo* investigations on other parasites and with other plant species extracts. Furthermore, combination of DE and plant leaf extracts may worth investigation in the future combining the mechanical damage of the parasites with the natural effect of some plant leaves.

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