El Colegio de la Frontera Sur

Estudio químico biodirigido de cuerpos fructíferos del hongo comestible: *Pleurotus eryngii* con actividad nematicida

TESIS

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Con orientación en Biotecnología Ambiental

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Dedicatoria

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I. Introducción

La ganadería en México tiene un impacto directo en la producción de alimentos de origen animal como carne y leche para contribuir al consumo nacional, además de beneficios por la generación de empleos directos e indirectos que sirven de sustento para las familias mexicanas. De acuerdo con la FAO (2015), en 2014 se estimó un total de 8, 575,908 cabezas de ovinos; asimismo, en 2015, el atlas agroalimentario nacional registró un inventario de 17.4 millones de ovinos y caprinos; además menciona que el Estado de Chiapas produjo 322, 087 cabezas de ovinos para carne (SIAP, 2016).

México posee las condiciones ideales para incrementar su producción y de esta manera satisfacer su demanda de carne y leche de ovino; no obstante, el potencial zootécnico y genético de sus animales se ha visto severamente afectado por factores de manejo de los animales y de los sistemas de producción (Cedillo-Martínez 2012).

Entre estos factores se encuentran las enfermedades y las parasitarias son unas de ellas, cuyas consecuencias se ven reflejadas en el deterioro de la salud del ganado con una importante repercusión económica para los productores. Las enfermedades parasitarias causadas por nematodos gastrointestinales (NGI) afectan la salud de los ovinos principalmente en las unidades de producción de tipo extensivo, donde el pastoreo constituye la base de la alimentación. En México, el impacto económico potencial anual en ganado bovino causado por NGI es de $ 445.10 millones de dólares (Rodríguez-Vivas *et al.*, 2017). El NGI *Haemonchus contortus*, es el parásito que mayores pérdidas económicas causa a nivel mundial en pequeños rumiantes (Laing *et al.*, 2013). En México,

su prevalencia en el sistema gastrointestinal de los ovinos es de hasta 37% (Aguilar-Marcelino *et al*., 2016).

Este parásito hematófago tiene un ciclo de vida rápido, los adultos se aparean en el abomaso del huésped y diariamente las hembras producen hasta 10 mil huevos que son excretados en las heces. Dentro del huevo se forma la primer larva (L_1), después de un día desarrolla y muda a L_2; una semana después, las larvas desarrollan a la fase infectante L_3, en esta etapa migran diariamente hacia el rocío del pasto y esperan a ser ingeridos por los rumiantes, una vez adentro, se dirigen al abomaso y a los intestinos, donde posteriormente desenvainan y mudan a L_4. Finalmente, en dos o tres semanas desarrollan a L_5 o adultos, obteniendo la madurez sexual con capacidad de reproducirse y comenzar de nuevo el ciclo (Schwarz *et al*., 2013; Laing *et al*., 2013).

El control de *H. contortus* se ha basado en el uso de químicos antihelmínticos (AHE), uno de los más usados es la Ivermectina (IVM), una lactona macrocíclica (LM) cuyo amplio espectro ha abierto las opciones para aplicarla en el tratamiento de parásitos del ganado, en peces de interés pecuario, mascotas e incluso humanos (Davies *et al*., 1998; Tišler y Kožuh Eržen, 2006; Garric *et al*., 2007); sin embargo, su uso frecuente y desmedido tiene dos desventajas muy importantes; la primera es la adquisición de resistencia antihelmíntica (RA) por parte de los parásitos, la RA está asociada a polimorfismos o mutaciones genéticas en moléculas diana de los parásitos, estos copulan y heredan la característica adquirida a nuevas generaciones, ocasionando que los mecanismos de toxicidad de los AHE no funcionen de forma adecuada (Laing *et al*., 2013; Andrioli-Salgado y de Paula-Santos, 2016).

En el Estado de Chiapas, específicamente en las comunidades Tzotziles-Tzeltales y en los municipios de Teopisca y Tapachula, Liebano-Hernandez *et al*. (2015) observaron RA de *H. contortus* presente en ovinos de la raza Chiapas y Pelibuey. Además, observaron que algunos ovinocultores de Teopisca y Tapachula desparasitan a los animales dependiendo de su condición corporal sin tener un programa de desparasitación regular.

La segunda desventaja, es el riesgo ecotoxicológico que representan los desechos AHE, debido a que afecta a otros organismos dependiendo del medio contaminado. Garric et al. (2007) señalan que alrededor de 45% del activo de IVM se libera en las heces dependiendo del animal tratado y la ruta de administración. Tišler y Kožuh Eržen (2006) coinciden con Beynon (2012) en que >98% del medicamento es excretado, mientras que Boxall *et al*. (2004), mencionan que los animales metabolizan LM entre 20-80%. Los efectos negativos de los residuos de IVM se han observado en organismos no objetivo de ecosistemas acuáticos y terrestres, sobre todo por el tiempo de vida media; en superficies su persistencia es breve, <30 días (He y Zhang, 2014), pero en sedimentos puede durar hasta 100 días (Tišler y Kožuh Eržen, 2006; Garric et al., 2007).

Los AHE eliminados en forma activa junto con las heces, ponen en riesgo a organismos benéficos como las moscas y escarabajos estercoleros (Martínez *et al*., 2011). Estos organismos son fundamentales para el equilibrio biológico de los pastizales y además, contribuyen al control de parásitos mediante la destrucción de huevos de nematodos contenidos en el estiércol (Martínez y Montes de Oca, 2013).

Por otro lado, Medina *et al*. (2014), indican que la dependencia de un solo método de control es ineficaz y poco sustentable a largo plazo y que la combinación de alternativas de control permite disminuir la dependencia de los fármacos. Por ejemplo, Hrckova y

Velebny (2013) mencionan que la combinación de dos AHE o la coadministración de drogas con nematicidas naturales de similar espectro de actividad o mecanismo de acción, son estrategias potenciales que han ayudado a disminuir el desarrollo de RA. Las principales estrategias para un manejo integral en el control de *H. contortus* son claramente descritas por Kearney *et al.* (2016). De la disciplina y responsabilidad con que se apliquen estas estrategias, dependerá la sustentabilidad y la rentabilidad en la producción de ovinos.

Las enfermedades parasitarias causadas por NGI requieren ser atendidas para reducir pérdidas económicas; deben tratarse de manera responsable con el ambiente, por tal motivo, es urgente buscar métodos complementarios ecológicos para disminuir el uso de los productos AHE. Una de las alternativas para el control de nematodos es el uso de hongos comestibles (HCM) productores de toxinas. De acuerdo a Li y Zhang (2014) los hongos son la mayor fuente de productos naturales biológicamente activos y la actividad nematicida es considerada una de las características de los hongos de este género. En basidiomicetos del género *Pleurotus*, se han aislado e identificado compuestos con actividad nematicida (ANT) contra algunos nematodos fitopatógenos (Palizi *et al.*, 2009), parásitos de ovinos (Pineda-Alegría, 2016) y nematodos de vida libre (Li *et al.*, 2001).

El primer compuesto nematicida aislado del género *Pleurotus* fue al ácido trans-2-decenedioico (Kwok *et al.*, 1992) y fue obtenido de *P. ostreatus*, de este hongo tambien se han aislado bioactivos nematicidas como el peróxido de ácido linoleico (Satou *et al.*, 2008) y proteasas (André-Genier *et al.*, 2015). En *P. pulmonarius*, se identificaron los compuestos: ácido S- coriolico, ácido linoleico, p-anisaldehido, p-anisyl alcohol, 1-(4-

methoxyphenyl)-1,2-propanediol y 2-hydroxy-(4'-methoxy)-propiophenone cuya ANT fue observada contra *Caenorhabditis elegans* (Stadler *et al*., 1993).

Dentro del grupo de hongos del género *Pleurotus*, se encuentra el HCM *Pleurotus eryngii*, un basidiomiceto con varias diferencias respecto a las demás especies; incluso, ha sido definido como un complejo debido a sus variaciones significativas de morfología, isoenzimas y características genéticas derivadas de las diferencias geográficas y ecológicas en el ambiente (Stajić *et al*., 2009). De acuerdo a Li et al. (2007), la actividad biocida de cheimonophyllon E; 5α,8α-epidioxyergosta-6,22-dien-3β-ol y del 5-hydroxymethyl-furancarbaldehyde fue observada contra nematodos de plantas, estos compuestos fueron identificados en una cepa de *P. ferulae* (ahora *P. eryngii* de acuerdo al Index Forungorum Partnership, 2017). La actividad nematicida del extracto acuoso de *P. eryngii*, fue observada en experimentos *in vivo* de ratones infectados con el cestodo *Hymenolepis nana* y el nematodo *Syphacia obvelata* (Samsam Shariat, Farid y Kavianpour, 1994).

La variedad de compuestos nematicidas identificados en otras especies de *Pleurotus* contra varios tipos de nematodos incluyendo gastrointestinales, sugiere que *P. eryngii* posee compuestos nemato-tóxicos contra *H. contortus*. Debido a esto surgen las preguntas de investigación ¿Los basidiomas de *P. eryngii* producen compuestos antihelmínticos contra *H. contortus*? ¿Cuál es la capacidad nematicida *in vitro* de los extractos orgánicos de *P. eryngii* contra este nematodo? En este sentido se ha planteado la siguiente hipótesis: "Los basidiomas de *Pleurotus eryngii* producen compuestos antihelmínticos contra el nematodo *Haemonchus contortus*". Para responder a la hipótesis se ha establecido el objetivo de "Evaluar *in vitro* la capacidad nematicida de

siete cepas del HCM *P. eryngii* para controlar al nematodo *H. contortus* a nivel de extractos acetónicos, hidroalcohólicos y de fracciones, así como identificar el o los compuestos involucrados en el efecto nematicida".

II. Capítulo de artículo: Molecular synergism between nematicidal compounds produced by *Pleurotus eryngii* against eggs and infective larvae (L_3) of *Haemonchus contortus*

Artículo sometido a la revista *Small Ruminant Research*

Dear Dr. Julio Cruz-Arévalo,

You have been listed as a Co-Author of the following submission:

Journal: Small Ruminant Research
Corresponding Author: LILIANA AGUILAR-MARCELINO
Co-Authors: Julio Cruz-Arévalo; José E Sánchez-Vázquez, Doctor; Pedro Mendoza-de-Gives, Doctor; Manases González-Cortazar, Doctor; Alejandro Zamilpa-Álvarez, Doctor; René Andrade-Gallegos, Master;
Title: Molecular synergism between nematicidal compounds produced by Pleurotus eryngii against eggs and infective larvae (L3) of Haemonchus contortus

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Molecular synergism between nematicidal compounds produced by *Pleurotus eryngii* against eggs and infective larvae (L_3) of *Haemonchus contortus*

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Abstract

The gastrointestinal parasite *Haemonchus contortus* is responsible for causing significant economic losses in sheep production. Control methods have been based on chemical anthelmintics (AHE), which has led to the development of anthelmintic resistance (AR) by the parasite. This study evaluated the *in vitro* AHE effect of seven strains of the edible mushroom (EM) *P. eryngii* against eggs and larvae (L_3) of *H. contortus*. The evaluation included acetone (AE) and hydroalcoholic (HA) extracts of the following strains: ECS-1138, ECS-1156, ECS-1255, ECS-1258, ECS-1261, ECS-1282 and ECS-1292. The HA extract of the strain ECS-1255 showed the highest mortality rate of L_3 (18.83%) at a concentration of 20 µg/mL. After subjecting this HA extract to normal phase chromatography column, five fractions were obtained; fraction F5 (100% MeOH) was the most effective against eggs, with hatching inhibition percentages of 88.77 and 91.87% at concentrations of 20 and 40 mg/mL, respectively. Gas Chromatography–Mass Spectrometry (GC-MS) subjected this fraction to an acetylation reaction to determine the content of secondary metabolites. The GC-MS analysis showed that the fraction F5 was composed of trehalose, polyols (L-iditol, galactitol, D-mannitol, D-glucitol and myo-inositol), adipic acid, stearic acid, squalene and β-sitosterol. The effectiveness of this fraction against eggs (the two highest concentrations showed no statistical differences with Ivermectin) suggests that it could be considered for *in vivo* evaluation as an anthelmintic alternative.

Keywords: Anthelmintics, polyols; *P. eryngii*; *H. contortus*; secondary metabolites

1. Introduction

The nematode *H. contortus* is a gastrointestinal parasite that affects small ruminants and causes major economic losses worldwide (Emery *et al.*, 2016). Infection begins when sheep ingest infective larvae (L_3) of nematodes present in grass dew, which provides these parasites with optimum moisture conditions for growth and survival (Kumarasingha *et al.*, 2016). At each stage of their life cycle, the high degree of genetic adaptability of these nematodes allows them to transcribe a large amount of genes related to their biological needs and environmental conditions, while their high prolificacy ensures the survival of large numbers of L_3 larvae (Laing *et al.*, 2013). In Mexico, the main method adopted by sheep farmers to control of *H. contortus* is chemical treatment (Aguilar-Marcelino, 2012). However, frequent and excessive doses of AHE have caused these parasites to develop AR, which they inherit to their offspring, allowing them to retain this ability (Medina *et al.*, 2014). Therefore, different alternatives to AHE have been implemented to control the populations of gastrointestinal nematodes in sheep. Several nemato-toxic compounds have been isolated and identified in edible mushrooms, such as fatty acids, alkaloids, peptide compounds, terpenes (Li *et al.*, 2007), condensed tannins, phenolic compounds (Ganeshpurkar *et al.*, 2012) and proteases (André Genier *et al.*, 2015). Until 2013, 23 species of *Pleurotus* genus mushroom had been shown to have nematicidal activity (NTA), which is considered a characteristic of this genus (Li and Zhang 2014). The EM *Pleurotus eryngii*, which has the common name of "king oyster", grows naturally in southern Europe, in areas of Central Asia and North America; it is regarded as the best of all *Pleurotus* species (Gaitán-Hernández, 2005). *P. eryngii* is described as a "species complex" due to the significant variations in morphology, isozymes and genetic

characteristics between specimens. These variations are derived from geographical and ecological differences in their environment (Stajić *et al*., 2009). The nematicidal properties of *P. eryngii* have been observed against *Syphacia obvelata* and the cestode *Hymenolepis nana*, reaching population reductions of 95% and 89%, respectively (Shariat *et al*., 1994). A study by Del Carmen *et al*. (2015) against larvae of *Ancylostoma caninum* reported *in vitro* mortality rates of 47.56%. Li *et al*. (2001), Mamiya *et al*. (2005) and Palizi *et al*. (2009) have described the NTA of *P. eryngii* against free-living and plant parasitic nematodes; however, has been little studied its effectiveness against *H. contortus*. Thus, the present study aimed to evaluate the AHE efficiency of seven strains of *P. eryngii* mushroom and identified by GC-MS the active compounds against eggs and L_3 larvae of *H. contortus*.

2. Materials and methods

2.1 Harvest of basidiomatas

The seven *P. eryngii* strains used in this study (Table 1) were grown on sterile substrate (a mixture of corncob, grass and sawdust, 65% moisture) according to the indications of Sánchez and Royse, (2001). Basidiomatas were produced at El Colegio de la Frontera Sur (ECOSUR), located in Tapachula, Chiapas, Mexico and were harvested when the pileus reached maximum extension and curvature; they were cut longitudinally and subjected to natural drying under the shade.

2.2 Collection, chemical fractionation and mycochemical characterization of extracts

The collection, fractionation and mycochemical characterization of extracts by Thin-Layer Chromatography (TLC) were carried out in the Phytochemical Department of Centro de Investigación Biomédica del Sur (CIBIS) of the Instituto Mexicano del Seguro Social (IMSS) located in Xochitepec, Morelos, Mexico.

2.2.1 Collection of acetone extracts

Dried mushrooms were crushed manually and macerated in acetone (≥99.5%, ACS) at 10:1 ratio (solvent: fruiting bodies); the same fruit bodies were soaked at three time points (24, 72 and 24 h) reaching three extracts at different time points. Each product of maceration was filtered by separated through a filter paper membrane (Whatman No. 4® qualitative filter paper) and then concentrated in a rotary evaporator (Laborota 4000, 45 °C/900 mbar/80 RPM) to obtain a remaining concentrated which was dried with a freeze dryer (Heto Drywinner) to obtain a lyophilized that was considered as acetonic extract (AE). Subsequently, an aliquot of the AE was dissolved in 10 mL of dichloromethane and was subjected to a qualitative TLC analysis. Aluminum slides precoated with silica gel 60 F_{254} and a mobile phase (95:5, *v/v*) prepared with dichloromethane (≥ 99.5%, ACS): methanol (≥ 99.8%, ACS) were used (Von Son de Fernex *et al.*, 2015). The spots were observed by short and long wave UV light and developed with $Ce(SO_4)_2$, ursolic acid purchased from Sigma-Aldrich (≥90%) was used as reference standard.

2.2.2 Collection of hydroalcoholic extracts

The dried mushrooms were submerged under ethanol (96%)-distilled water solution (60:40, *v/v*) during 24 h, at same ratio of AE. The liquid obtained was filtered and concentrated in a rotary evaporator (55 °C/900 mbar); the remaining fraction was lyophilized to obtain the dry hydroalcoholic extract (HA). An aliquot was dissolved in 10 mL of MeOH and then was subjected to TLC analysis; the mobile phase and chemical developer were α-naphthol (≥ 99%) and $Ce(SO_4)_2$, the reference compounds were oleanolic acid (≥93.22%) and β-sitosterol-D-glucoside (≥75.0%); both purchased from Sigma-Aldrich, the spots were also visualized by short and long wave UV light.

2.2.3 Column fractionation of hydroalcoholic extract of ECS-1255 strain

The extract obtained from *P. eryngii* ECS-1255 strain showed the highest degree of nematicidal activity compared to the other strains, and was fractionated using open-column chromatography. A chromatographic column was packed up with 35 g of silica gel (70-230 mesh, Merck) normal phase. The extract (3.6 g) was adsorbed on 5 g normal phase silica and added to the column; fractionation was performed by three eluent dichloromethane-methanol systems (CH_2Cl_2-MeOH: 90:10, 70:30, 50:50 *v/v*); and the column was washed with MeOH J.T.Baker® (≥ 99.8%). Four fractions of 250 mL were collected from each system (a total of 1 L); however, a precipitate was formed during last collection from 70:30 system, so it was also considered as a fraction; thus, five fractions (F1-F5) were collected. Each eluted fraction was concentrated with the same parameters used for AE and then were lyophilized and stored in glass vials. TLC analysis of F5 fraction

dissolved in MeOH was performed using a butanol (99.4%)-acetic acid (≥ 98%)-distilled water system (3:5:2 *v/v*), the spots were stained with α-naphthol and visualized by UV light.

2.3 *In vitro* bioassays

The eggs and larvae collection as well as the *in vitro* bioassays were done at the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), in the department of Helminthology of the Centro de Investigación Disciplinaria en Parasitología Veterinaria (CENID-PAVET) located in Jiutepec, Morelos, Mexico.

2.3.1 Collection of L_3 larvae and eggs of *H. contortus*

A healthy male sheep (<3 months) was infected with larvae of *H. contortus* at a dose of 350 L_3/kg live weight. Feces containing parasite eggs were collected 28 days later. A stool culture was prepared in a basin at room temperature and with adequate moisture and ventilation. After seven days, L_3 larvae were recovered using the Baermann larval migration technique and then were washed and the sheath was removed according to methods described by Liébano-Hernández *et al*. (2011). The eggs were obtained by taking a stool sample directly from the rectum of the sheep; they were subsequently purified according to the technique used by Pineda-Alegría, (2016). The treatment of the sheep was carried out following the indications of the Directive 2010/63/EU and the mexican standards (NOM-051-ZOO-1995 and LEY FEDERAL DE SALUD ANIMAL).

2.3.2 Assessment *P. eryngii* extracts against L_3 larvae of *H. contortus*

The *in vitro* confrontation was performed five times conducted by the indications of Table 1. The confrontation mixture between extract and 100 L_3 larvae were placed in 0.2 mL microtubes and stirred with a vortex to ensure homogeneity. The tubes were covered and incubated for 72 h at 28 ± 1 °C. The AHE effect was measured by placing 10 µL aliquots from the mixture on a slide and counting dead larvae using a compound microscope 10X objective. The criterion for distinguishing live and dead larvae was motility and the response to physical stimuli.

The AHE effectiveness was calculated using the following formula:

% of mortality= (a- b)/(100- b) x 100

Where: a= mean of the treatment and b= mean of the negative control

(Belemlilga-Bonewendé *et al.*, 2016).

2.3.3 *In vitro* evaluation of the F5 fraction against eggs and L_3 larvae of *H. contortus*

A previous bioassay (data not shown) of the five fractions against L_3 larvae was performed to evaluate which was more effective, F5 fraction resulted the better. *In vitro* bioassays against larvae and eggs were carried out on Elisa plates. Concentrations of F5 fraction were 3, 5, 10, 20 and 40 mg/mL against eggs and larvae. The controls for confrontation against eggs were IVM at 1.75 mg/mL and DMSO 2.5% and controls for larvae were IVM at 5 mg/mL and MeOH 4%. In both cases, concentrations were adjusted from a solution of F5 fraction at 80 mg/mL dissolved in MeOH 8%.

The faecal egg count was performed using the McMaster technique (Aguilar-Marcelino, 2012); afterwards, the mixtures were prepared for the confrontation with 50 µL of each treatment and 50 µL of aqueous suspension containing 100 eggs of *H. contortus.* Mixtures were kept at 28 ± 1 °C during 72 hours. The egg hatching inhibition test was made by taking aliquots of 10 µL from the mixtures and formation of L_1 larvae was observed using a compound microscope (10X). The hatching inhibition percentage was determined by the same formulae used in the extracts bioassays.

2.3.4 Statistical analysis

The mortality and inhibition effectiveness was compared using a completely randomized design for ANOVA LSD test for extracts and Tukey's test for fraction with means comparison (α= 0.05) using the statistical software "R", version 3.2.1 (2016). In addition, the median lethal concentration (LC_{50}) was determined by Probit analysis fitted to a generalized linear model.

2.4 Derivatization of the F5 fraction

The F5 fraction was subjected to an acetylation reaction according to Kitson *et al*. (1996) with some modifications. Three mL of acetic anhydride were added to 10 mg of F5; one mL of pyridine was added as catalyzer and after 15 minutes, the reaction was stopped with ice. The crude reaction mixture was supplemented with ethyl acetate, which yielded two phases. The fraction containing the compounds (FRAcet) was concentrated in a rotary evaporator (45 °C/900 mbar/80 RPM). Subsequently, the acetylated fraction was

absorbed on silica gel (70-320 mesh) and eluted with CH_2Cl_2 100% and CH_2Cl_2: MeOH (90:10) systems on a glass column with normal phase silica gel. The two subfractions obtained (EXT94 and EXT95) were sent to the Centro de Investigaciones Químicas de la Universidad Autónoma del Estado de Morelos (CIQ-UAEM) for analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

2.5 GC-MS analysis of F5 fraction

GC-MS analysis of EXT94 and EXT95 subfractions was performed using an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N Mass Selective Detector (MSD) with ionization energy (70eV). A HP-5MS column (30m x 0.250mm id., 0.25-μm film thickness, coated with 5% dyphenyl and 95% dimethylpolysiloxane) was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min and an injection volume of 1 μL was employed. The temperature of the injector was 250 °C and the Ion-source temperature was 230 °C. The oven temperature was kept at 40 °C for 1 min, then the temperature was programmed at 5 °C/min to 250 °C, which was maintained for 1 min, finally the temperature was increased 10 °C/min to 285 °C and was kept for 20 min.

3. Results

3.1 Mycochemical profile of extracts and F5 fraction

The compounds present in both extracts were qualitatively analyzed by TLC; the chemical reaction with $Ce(SO4)_2$ suggests presence of several phenolic compounds in the AE. All strains had the same chromatographic profiles aligned on the slides. In the case of HA

extracts, most of the compounds did not migrate with the mobile phase CH_2Cl_2:MeOH (95:5); however, spraying the slides with $Ce(SO_4)_2$ showed that profiles of the strains aligned with the migration profile of oleanolic acid. The Rf value of oleanolic acid was 0.57 while the value profiles of HA extracts was 0.60. The presence of glucosidic compounds was observed as well as possible terpenes that shared profile with β-sitosterol glucoside.

3.2 Observing the anthelmintic effect of extracts

Nematicidal activity was more effective for HA than AE. The ECS-1255 strain was the only that showed activity in both extracts (Table 3), difference was statistically significant compared to other strains of the AE group; however, this value was lower compared to the IVM. Regarding HA extracts, the ECS-1255 strain had the greatest nematicidal effect with 18.83%, although there was no statistical difference compared with strains ECS-1258, ECS-1156, ECS-1292, and the effect was significantly lower than the control with IVM ($p= <0.0001$).

3.3 Effect of the F5 fraction against larvae and eggs of *H. contortus*

The F5 fraction showed no effect on larvae mortality (data not shown); however, the larvae confronted with this fraction showed little motility, presented intern ulcers and contractions (Figure 1a) compared with negative control larvae, which looked very active and without physical damage (Figure 1b). In eggs confrontation, the F5 fraction showed hatching inhibition percentages of 42.78, 48.56, 73.52, 88.77 and 91.87% for concentrations above mentioned, respectively. Several eggs and L_1 larvae were completely distorted in

treatments with F5 fraction (Figure 1d, 1e) while in negative control L_1 larvae got hatched (Figure 1f). There was no statistically significant difference between effectiveness of positive control (IVM 1.75%) and F5 fraction at concentrations of 40 and 20 mg/mL (Figure 2). The calculated LC_{50} was 4.19 mg/mL, and the upper and lower confident limits were 4.60 and 3.12 mg/mL, respectively.

3.4 Compounds identified in the F5 fraction by GC-MS

Interpretation of mass spectrum was conducted using the database of National Institute Standard and Technology (NIST Version 1.7). The identified compounds of subfractions are shown in Figure 3 and 4, their structures are illustrated in Figure 5. The compounds in the EXT95 subfraction were trehalose (73.36%), polyols (glucitol and myo-inositol) with 17%, adipic acid (6.33%), stearic acid (0.25%), squalene (0.41%) and β-sitosterol (1.02%). In EXT94 subfraction, trehalose constituted 65.71%, polyols (L-iditol, galactitol, D-mannitol and D-glucitol) accounted for 24.20%, β-sitosterol for 5.05% and adipic acid was present at 4.22%.

4. Discussion

The AHE *in vitro* of extracts obtained from the mycelium of *P. eryngii* against *H. contortus* L_3 has already been evaluated; however, the effect of basidiomatas extracts was unknown. In a study by Comans-Pérez, (2014), the HA mycelium extracts from *P. eryngii* ECS-1292 strain caused 95% L_3 mortality after 48 h *in vitro* confrontation. The efficiency percentage of ECS-1255 strain is similar to *in vitro* study reported by Huicochea Medina

(2015), who evaluated 400 µg/mL of HA extracts from *P. eryngii* mycelium (ECS-1292 strain) against L_3, after 24 h of confrontation observed 16.6% of rate mortality. The similarity between these results indicates that biological state of mushroom influences the amount of nematicidal metabolites present in the extract; it is presumed that higher concentrations of carpophores extract, yield better mortality rates. Moreover, it is possible that drying the mushroom influences the amount of metabolites. The amount of flavor components varied when Li *et al*. (2015) evaluated *P. eryngii* mushrooms under different drying methods.

HA extracts caused physical damage to the larvae, they had intern ulcers in their body, which were probably caused by HA extracts absorption through transcuticular diffusion. HA extracts from *Croton macrostachyus* seeds, *Ekebergia capensis* and *Hedera helix* plants showed NTA values between 60 and 100% against eggs and larvae of *H. contortus* (Eguale *et al*., 2007, 2006). These authors attribute the effect to the easiness transcuticular absorption of HA extracts; furthermore, they mention that most AHE drugs enter the body of nematodes, tapeworms and trematodes through this pathway. Harder (2016) indicates that *H. contortus* absorbs AHE drugs such as Levamisole and Macrocyclic lactones through the cuticle. The AHE activity of HA extracts has also been observed against phytonematodes. Oka (2012) obtained the best nematicidal effect against *Meloidogyne javanica* with aqueous HA extracts from *Verbesina encelioides* flowers. This suggests that HA extraction is a good alternative for obtaining efficient nematicidal metabolites.

The high percentages of egg hatching inhibition indicate that methanolic fraction of the extract from *P. eryngii* ECS-1255 strain has nematicidal compounds. The variety of

glucide compounds present in the fraction suggests that they could be responsible for the ovicidal effect. According to Oka (2012), glucoside compounds have nematicidal properties. However, there is no certainty that trehalose as major component of the fraction is responsible for some AHE effect; in fact, this sugar is necessary for survival of nematodes under water stress and freezing conditions (Perry and Wharton, 2011). Trehalose also plays a fundamental role during larval development within both the egg and physiology of nematodes (Lee, 2002). Although trehalose has been observed to inhibit egg hatching by osmotic pressure, this effect is temporary and reversible, since expelling trehalose through the permeable membrane allows the larvae to continue their development.

Five polyols (Figure 5) were identified in the present study; four of them have aliphatic structure, very similar to xilitol while myo-inositol is a cyclic polyol. It is likely that these polyols are responsible for the ovicidal effect. Compounds with analogue structures to nematicidal molecules might have some nemato-toxic effect. Marie-Magdeleine *et al.* (2011) mention that the nematicidal properties of cucurbitina amino acid are due to the similarity of its structure with the kainic acid AHE. Seo et al. (2014) observed nematicidal activity of natural ester compounds and their analogues with high percentage activity against *B. xylophilus*. The embrionary development of the silkworm *Bombyx mori* was inhibited by sorbitol (D-glucitol) polyol; interestingly, trehalose also had a detriment role in embrionary development (Horie *et al.*, 2000).

An older study showed that egg hatching of *M. incognita* was inhibited by a synthetic polyol over 23% of concentration in a semisolid phase; moreover, some juveniles (J2) larvae were found distorted (Ko and Van Gundy, 1988). In other survey, Cedillo-Rodríguez

(2016), obtained 100% of hatching inhibition of *H. contortus* eggs exposed *in vitro* to a fraction containing 1.25 mg/mL of xylitol; it is worth noting that the LC_{50} obtained in this fraction was 4.19 mg/mL, a similar concentration to that obtained in the present study. Xylitol is known by its bioactive properties as antimicrobial effects and benefits for dental health (Kumar *et al*., 2008). Moreover, Nilsson *et al*. (2011) mention that xylitol showed inhibition in children's ear infection. In general, polyols are compounds with several hydroxyl functional groups, Park *et al*. (2007) reported stronger nematicidal activity of compounds with hydroxyl or methoxy groups against *B. xylophilus* nematode. This could support the hypothesis that polyols are the principal bioactive compounds against *H. contortus* eggs in present study.

The β-sitosterol was also detected in the F5 fraction; it is known that this compound can be conjugated with sugars. This sterol may have formed complexes with the glucide carbohydrates in the F5 fraction and thus acted synergistically as AHE; for example, the glucoside β-sitosterol had a maximum effective concentration (EC_{50}) of 82.50 µg/mL when confronted against the nematode *Caenorhabditis elegans* (Deepak *et al*., 2002). In a revision of Ohri and Pannu (2009) it is pointed out that glycoside compounds varied in mortality rates of *M. incognita* nematode. Oka (2012) mention that glycosides are known by nematicidal properties.

Hrckova and Velebny (2013) also document in several reports, the anthelmintic effect of glycoside compounds. Acording to Park *et al*. (2007), several compounds as alcohols, fatty acids derivatives, aldehydes, terpenoids and phenolics can act sinergistically or independently as insecticidal or nematicidal agents. Conjugation of sugars may also

explain the difficulty of migration compounds on the silica plates and the chromatographic separation.

Stearic acid was also detected in the F5 fraction, the AHE property of fatty acids is already known, acording to Degenkolb and Vilcinskas (2016), linoleic acid is noted as the nematicidal principle of nematophagous trap-forming fungi. The nematicidal activity of stearic acid has been observed against *C. elegans* (Stadler *et al*., 1993), *M. incognita*, *Aphelencoides besseyi* and *Panagrellus redivivus* (Ghisalberti, 2002). The structure of adipic acid is also very similar to that of fatty acids; however, according to Nagase *et al*. (1982), there was not effect when it was evaluated against *B. lignicolous*; so, the nematicidal effect against *H. contortus* could be unlikely.

Squalene is a triterpene that was also identified in the F5 fraction; terpenes are widely known for their nematicidal effect (Ohri and Pannu, 2009). The bioactive properties of squalene have been studied in terms of its cytotoxic and anti-malarial effects (Sangsopha *et al*., 2016). Seven different triterpenes caused rates of mortality between 10 and 80% in *M. incognita* (Begum *et al*., 2015). Terpens as ursolic acid and β-sitosterol showed 70 and 60% effectiveness when were confronted against *M. incognita* phytonematode, respectively (Ferheen *et al*., 2011). However, although squalene is a triterpene, their nematicidal effect was not observed when was confronted against *H. contortus* L_3 larvae (data not shown).

Since there were no significant differences between concentrations of 20 and 40 mg/mL and IVM, the F5 fraction might be considered as an AHE to be evaluated in models *in vivo*. According to the standards of the WAAP (World Association for the Advancement of

Veterinary Parasitology) for rating anthelmintic, a 90% efficacy is considered very good while a 80-90% is moderately effective (Wood *et al.*, 1995).

5. Conclusions

Unlike other species of the genus *Pleurotus*, the nemato-toxic compounds of *P. eryngii* had not yet been identified. This study demonstrates the presence of several compounds in *P. eryngii* whose nematicidal effect had been previously reported; this proves that the fruiting bodies of *P. eryngii* have nemato-toxic metabolites. Furthermore, we identified compounds with no previous report of NTA but with similar structures to natural or synthetic nematicidal compounds that could be considered for evaluation as alternative AHE. The present study identified five different polyols, whereas only three have been typically reported in strains of the genus *Pleurotus*. The assessment of polyols identified (individually or in combinations) as inhibitors of *H. contortus* egg hatching is a possibility to be taken into account for the control of this parasite. If the results are successful, then *P. eryngii* mushroom is a potential option to produce these metabolites. However, the control of *H. contortus* with toxins derived from mushrooms or any other natural alternative should not be considered as the unique option; a combination of several strategies must be carried out as an integral control to avoid the development of any type of resistance or immunity by the parasite.

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Figures

Table 1. Confrontation of extracts of *P. eryngii* against *H. contortus* larvae L_3.

Extract or control	*P. eryngii* strain (Treatment)	Concentration	*H. contortus* larvae
AE	ECS-1138 ECS-1292 ECS-1258 ECS-1156 ECS-1255 ECS-1261 ECS-1282	20 μg/mL	100 L_3 in 80 μL of distilled water
HA	ECS-1138 ECS-1292 ECS-1258 ECS-1156 ECS-1255 ECS-1261 ECS-1282	20 μg/mL	
MeOH[1]	-	4%	
IVM [2]	-	10 mg/mL	

n=5 Methanol[1] (Negative control) IVM[2] (Positive control)

Table 2. Principal compounds observed on TLC plates.

Acetonic extracts	Hydroalcoholic extracts
Fatty acids[a]	Terpens[ab]
Coumarins[a]	Primary metabolites[ab]
Terpens[a]	Glucid compounds[b]
Chromons[a]	

a) Developed with $Ce(SO_4)_2$ b) Developed with α-naphthol

Table 3. Death rate of *H. contortus* L_3 larvae against hydroalcoholic and acetonic extracts of *P. eryngii* edible mushroom.

Treatment	Hydroalcoholic extracts		Acetonic extracts	
	% of mortality ± sd	Group	% of mortality ± sd	Group
ECS-1255	18.83 ± 3.49	b	9.63 ± 2.44	b
ECS-1258	17.67 ± 5.73	bc	1.95 ± 2.47	cd
ECS-1156	15.46 ± 5.62	bcd	3.72 ± 2.82	cd
ECS-1292	13.64± 5.37	bcd	0.76 ± 2.02	d
ECS-1261	12.65 ± 7.66	cd	3.54 ± 3.53	cd
ECS-1138	12.47 ± 2.71	cd	1.50 ± 2.47	cd
ECS-1282	11.55 ± 5.69	d	2.30 ± 2.95	cd
IVM	100 ± 00	a	95.56 ± 0.00	a
MeOH	0 ± 00	e	4.43 ± 0.91	c

n=5 72 hours post-confrontation 28°C *Groups with different letters indicate significant difference ($p<0.05$) ANOVA and LSD test.

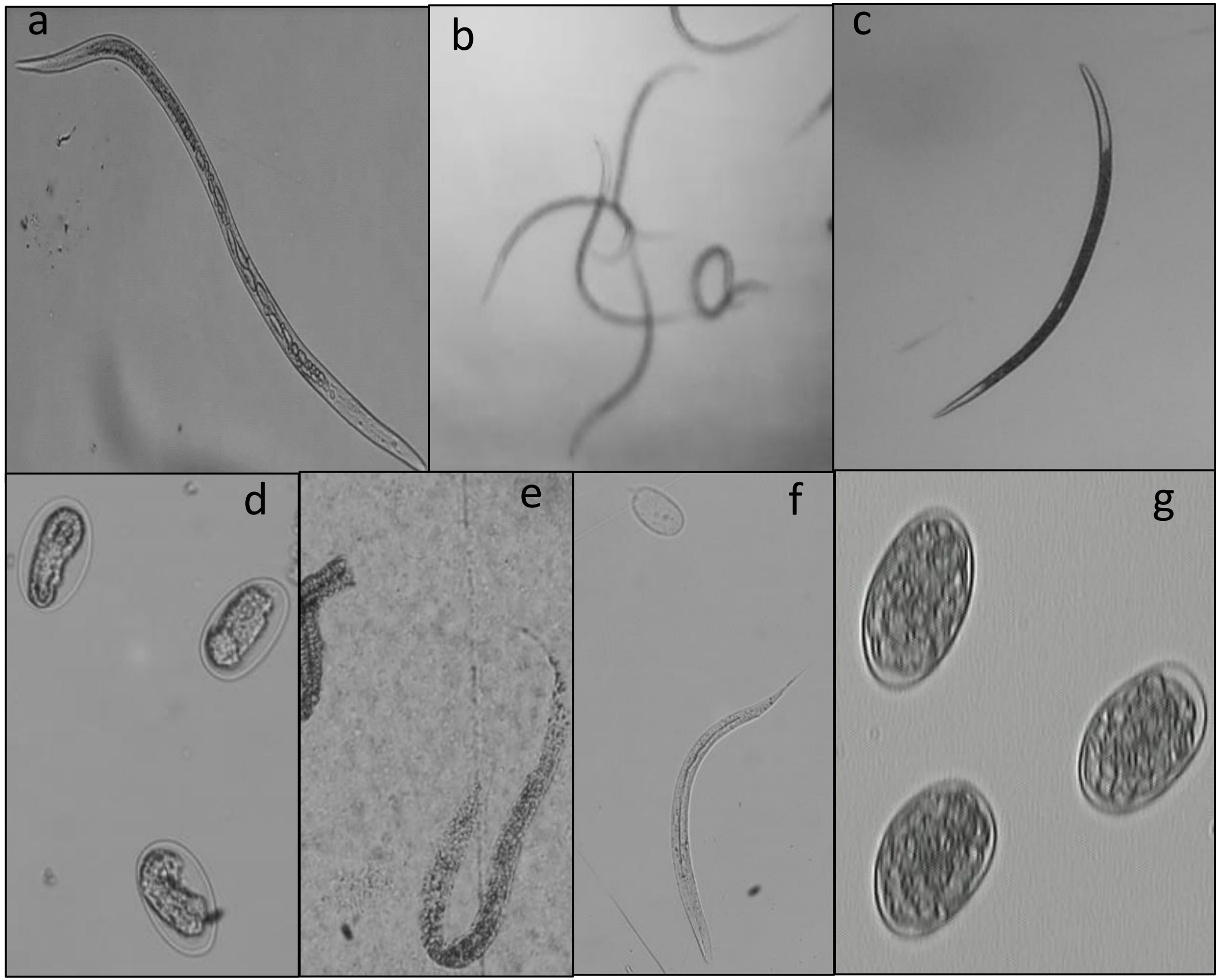

Figure 1.Eggs and larvae damaged by F5 fraction. a) Larva with intern ulcers; b) Active larvae of negative control; c) Dead larva in positive control (IVM); d) Larval development inhibited in eggs; e) A dead L_1 larva that hatched but dit not survive; f) A L_1 larva that hatched in negative control; g) Eggs hatching inhibited by positive control (IVM)

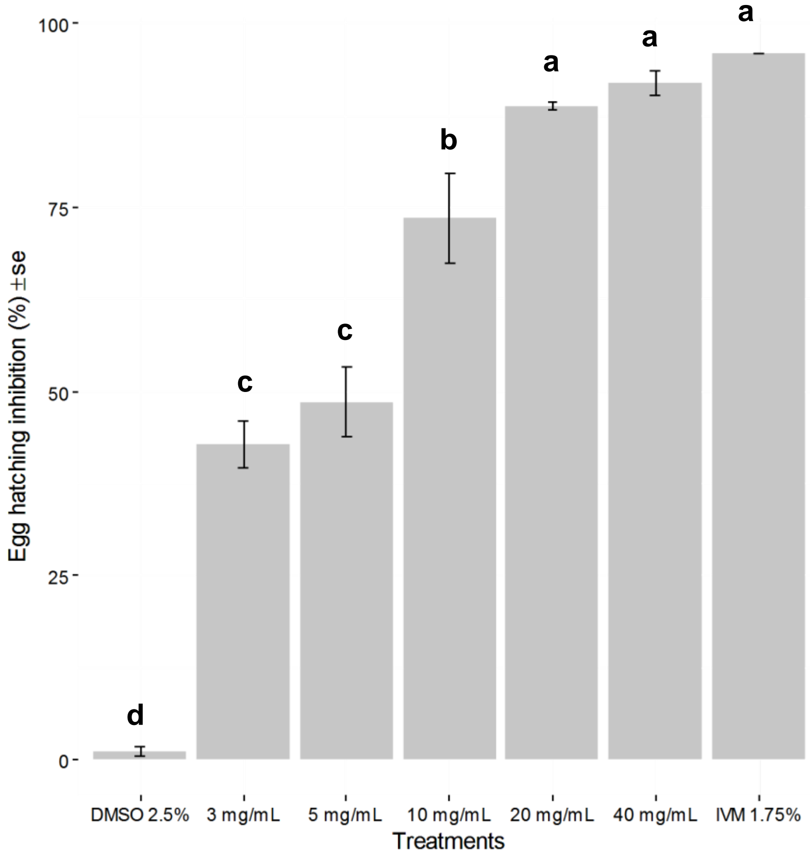

Figure 2. Effectiveness of the F5 fraction on eggs hatching inhibition of *H. contortus*.

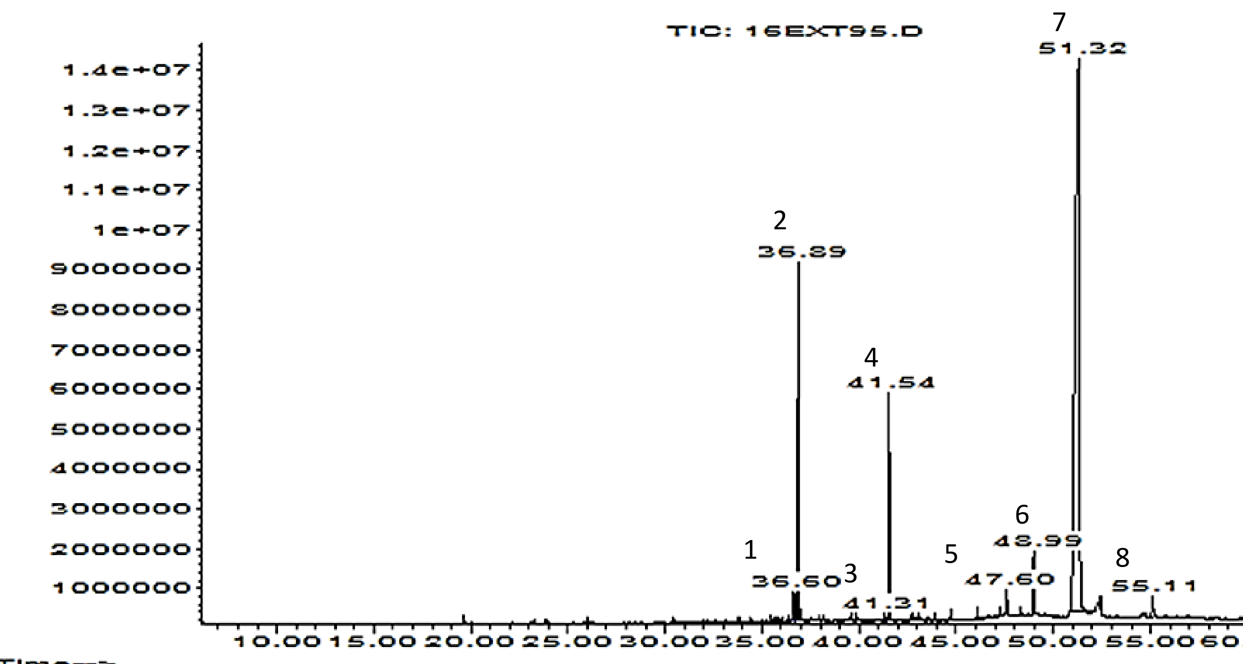

Figure 3. GC-MS of the first subfraction. Peaks:1) Myo-inositol, 2) D- Glucitol, 3) Stearic acid, 4) Adipic acid, 5) Squalene, 6) nd (not detected), 7) Trehalose, 8) β-sitosterol

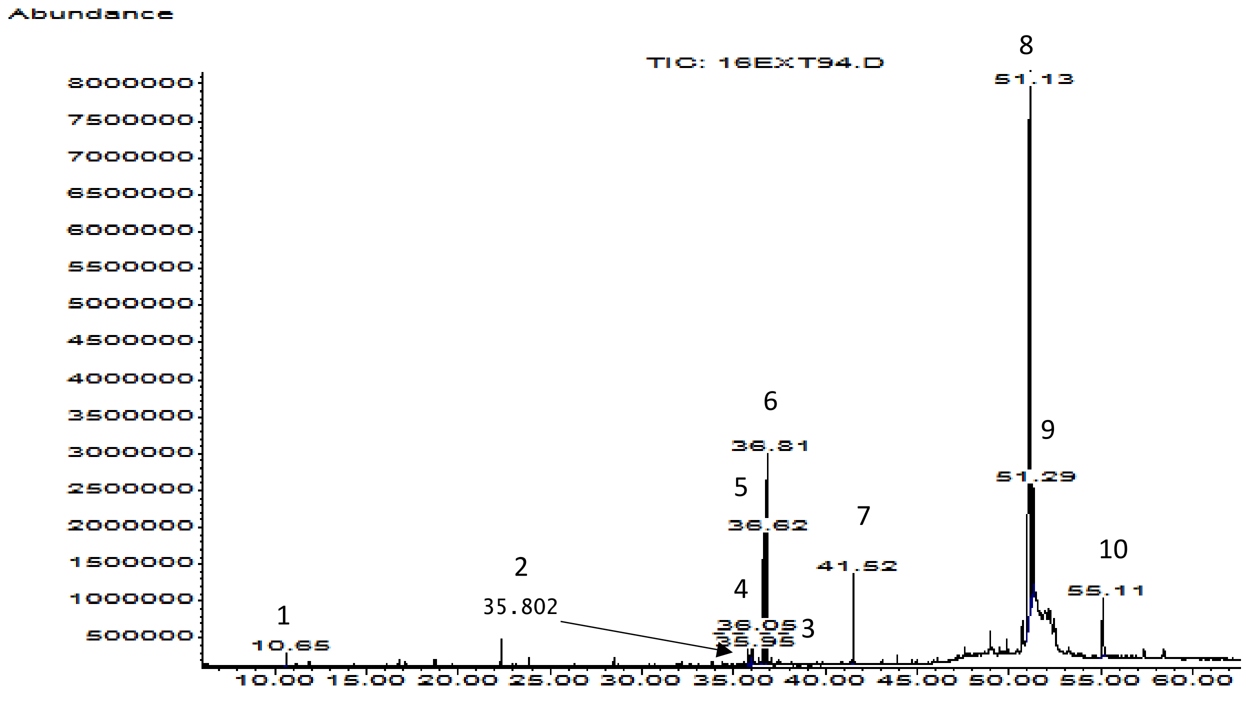

Figure 4. GC-MS of the second subfraction. Picos: 1) Propanol, 2) Galactitol, 3) L-Iditol, 4) L-Iditol, 5) D-Glucitol, 6) Mannitol, 7) Adipic acid, 8) Trehalose, 9) Trehalose, 10) β-sitosterol.

Figure 5. Chemical structures of the compounds identified in fraction F5. L-Iditol taken from PubChem; Open Chemistry Database, the other compounds were taken from the National Institute of Standards and Technology (NIST 69) database.

III. Conclusiones

En el presente trabajo se evaluó la actividad nematicida de extractos hidroalcohólicos y acetónicos de cuerpos fructíferos de siete cepas del hongo comestible *P. eryngii*, los resultados observados durante el presente estudio permiten determinar lo siguiente.

- Sin importar las variaciones morfológicas entre algunas cepas, el análisis TLC sugiere que debido a la similitud entre los perfiles cromatográficos, todas las cepas poseen los mismos compuestos o similitud entre ellos.
- La actividad nematicida del extracto hidroalcohólico de la cepa ECS-1255 no fue estadísticamente diferente respecto a las cepas ECS-1258, ECS-1156 y ECS-1292; sin embargo, la alta producción de basidiomas y el buen rendimiento del extracto hidroalcohólico colocan a la cepa ECS-1255, como una opción potencial para estudios posteriores, por ejemplo contra larvas L_4 o la evaluación *in vivo* de la fracción F5 en pruebas de laboratorio con gerbiles o directamente en ovinos.
- La fracción metanólica F5 obtenida mediante cromatografía en columna, fue la más efectiva en la inhibición de la eclosión de huevos de *H. contortus* con un porcentaje de 91.8% a dosis de 40 mg/mL, de acuerdo a los estándares de la WAAVP (Asociación Mundial para el Avance de la Parasitología Veterinaria, por sus siglas en inglés) una eficacia antihelmíntica del 90% se considera muy buena, por lo que la fracción F5 entra dentro de esta categoría, un porcentaje entre 80-90%, moderadamente efectiva. En consecuencia, se requieren mayores estudios para observar la eficacia de la fracción F5 en modelos *in vivo*.
- En esta fracción se identificaron compuestos cuyo efecto nematicida ya ha sido previamente registrado; sin embargo, también se detectaron los polioles, que son

candidatos para evaluar su efecto AHE debido a su similitud en estructura con el Xilitol, un poliol que ha demostrado alta inhibición de la eclosión de huevos en modelos *in vitro*. La evaluación del escualeno como agente nematicida fue realizada por primera vez en el presente trabajo, no se observó algún efecto que pueda considerarse como negativo para el nematodo. Además, cabe destacar que la fracción F5 no causó la mortalidad de larvas L_3, sin embargo; se observó una disminución en la motilidad, efecto que al paso del tiempo podría influir en el desarrollo normal de la larva y provocar su muerte por inanición.

- Cabe destacar que en la presente investigación, se observó que la cepa ECS-1255 posee cinco diferentes polioles, de acuerdo a la literatura, el manitol es el más común y generalmente en otros estudios se ha reportado la presencia de no más de tres diferentes polioles en una misma cepa de *P. eryngii*.
- Las observaciones previamente mencionadas convierten a *P. eryngii* como una alternativa para el control del parásito *H. contortus* y aportan evidencia de su efecto nematicida contra NGI, debido a que la mayoría de estudios nematicidas realizados con este HCM se han ensayado contra fitonematodos.
- Los compuestos cuya efectividad nematicida ya ha sido reportada y que se identificaron en la fracción F5 permiten concluir que los basidiomas de P. eryngii si producen metabolitos nematicidas, lo que conduce a aceptar la hipótesis planteada.
- Se espera que las nuevas condiciones del cambio climático, favorezcan la persistencia de *H. contortus* durante temporadas más prolongadas, afectando de esta forma la producción del ganado; en consecuencia, es necesario diseñar

mecanismos que permitan afrontar la problemática y mantener niveles aceptables de producción. Se recomienda llevar a cabo un manejo integral mediante la combinación de diferentes estrategias como la rotación de pastoreos, pastoreo mixto, vacunas, mejoramiento genético, nutrición, hongos nematófagos, hongos productores de toxinas, dosificación moderada de medicamentos antihelmínticos, uso de hierbas, etc. Estas estrategias permitirán controlar las poblaciones de nematodos de ovinos de una manera sustentable y responsable con el medio ambiente.

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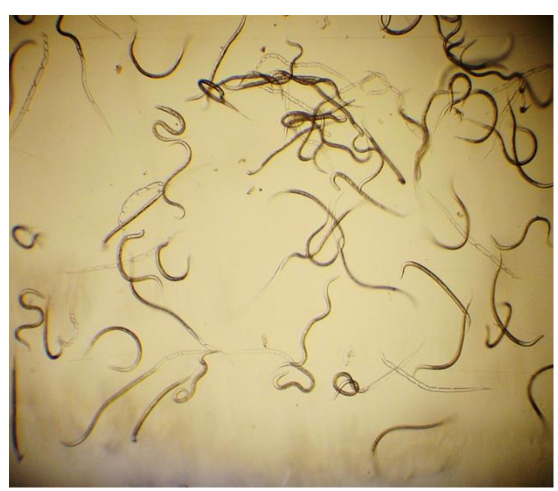
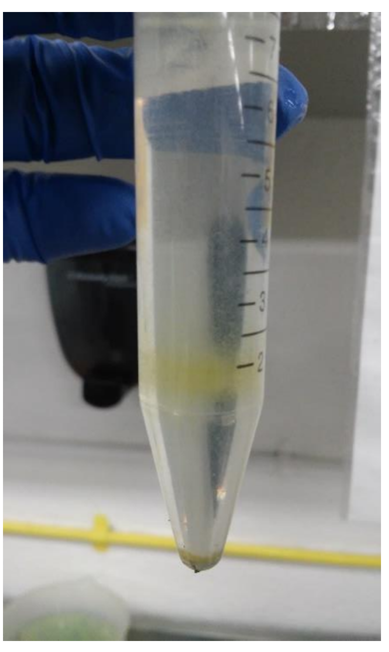
V. Anexos

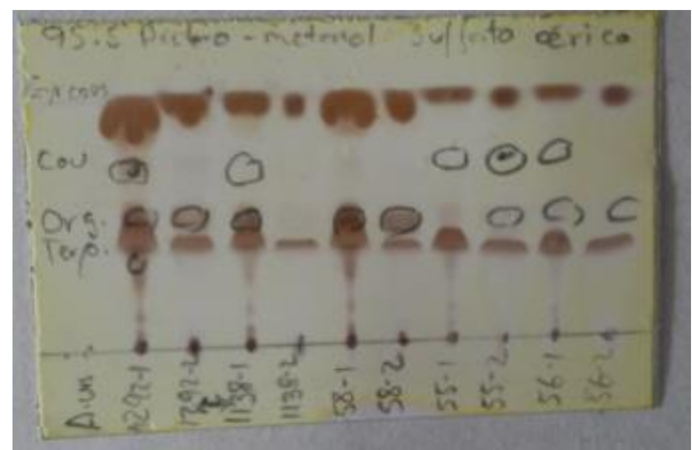
Anexo 1. Basidiomas secos del hongo comestible *P. eryngii* ECS-1255



Anexo 2. Larvas en proceso de desenvaine con estimulado con hipoclorito de sodio



Anexo 3. Paquete de huevos de *H. contortus* formando un gradiente en solución salina.



Anexo 4. Cromatoplacas de extractos acetónicos revelados con $Ce(SO_4)_2$

FECHA DE LECTURA: 23/06/2016, 11:00 h — inicio: 20/06/2016 13:00 h

HORAS DE LECTURA: 72

huevo de H. contortus vs Fracción F5 de P. eryngii en metalol acuoso (4%)

	DMSO 2.5%								IVM 1.75 5								40 mg/mL							
	H	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**
1	0	6	0	13	1	4	1	22	7	0	14	0	16	0	19	0	11	2	4	0	8	1	6	0
2	0	18	1	25	0	6	0	6	10	0	8	0	17	0	10	0	10	0	10	0	7	0	11	0
3	0	6	0	20	3	36	0	4	11	0	8	0	15	0	12	0	7	0	13	0	15	1	7	0
4	0	31	1	9	0	37	1	43	6	0	7	0	18	0	14	0	7	1	6	0	13	0	10	0
5	0	5	0	1	0	3	1	7	13	0	8	0	12	0	18	0	7	0	8	0	8	0	5	0
6	0	3	1	5	1	6	0	11	8	0	2	0	1	0	1	0	8	1	3	0	7	0	7	0
7	0	1	0	2	1	4	1	3	6	0	2	0	8	0	5	0	3	0	4	0	4	0	5	1
8	0	6	0	34	0	2	0	3	12	0	11	0	11	0	6	0	3	1	13	0	10	1	12	1
9	0	8	0	15	1	3	2	6	16	0	16	0	9	0	15	0	4	0	4	1	6	0	4	0
10	0	6	3	8	1	9	1	26	9	0	11	0	11	0	9	0	1	1	3	0	7	0	11	0
TOTAL	0	90	6	132	8	110	7	131	98	0	87	0	118	0	109	0	61	6	68	1	85	3	78	2
SUMA V	90		138		118		138		98		87		118		109		67		69		88		80	
PORCEN	0	100	4.35	95.7	6.78	93.2	5.07	94.9	100	0	100	0	100	0	100	0	91	8.96	98.6	1.45	96.6	3.41	97.5	2.5
PORCEN	4.049987718				95.95001228				100				0				95.92160246				4.078397538			
PROM Y	4.05	2.89							100	0							95.9	3.35						
				484	INDIVIDUOS MUESTREADOS							412								304				

Anexo 5. Datos crudos del bioensayo de la fracción F5 contra huevos de *H. contortus.*

	20 mg/mL								10 mg/mL								5 mg/mL							
	H	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**	**H**	**L**
1	13	1	14	2	11	1	9	1	15	2	3	3	5	5	9	3	5	11	7	11	9	6	9	12
2	23	3	15	1	10	1	15	1	8	0	3	1	8	5	10	4	2	9	8	4	7	7	5	10
3	16	1	19	0	14	2	11	1	5	1	6	0	10	4	11	7	8	7	5	2	2	1	10	9
4	13	2	14	0	13	1	7	0	9	0	8	2	7	4	11	6	2	10	6	5	4	4	5	7
5	7	1	9	1	10	1	14	0	11	1	6	0	9	3	7	1	4	5	6	2	7	16	3	6
6	9	0	0	0	6	0	2	0	9	0	10	1	2	1	8	2	5	2	4	1	3	3	5	3
7	16	1	4	0	17	0	11	0	14	1	8	1	6	4	5	2	6	3	9	3	4	4	8	0
8	16	1	9	1	5	1	7	2	4	1	11	4	3	0	5	3	5	6	10	10	4	6	12	4
9	9	1	19	2	15	2	7	1	10	0	14	1	9	5	6	3	6	10	7	2	6	3	10	5
10	0	0	7	0	12	1	6	0	0	2	6	1	6	4	6	3	6	6	10	0	7	5	8	4
TOTAL	122	11	110	7	113	10	89	6	85	8	75	14	65	35	78	34	49	69	72	40	53	55	75	60
SUMA V	133		117		123		95		93		89		100		112		118		112		108		135	
PORCEN	91.73	8.27	94	5.98	91.9	8.13	93.7	6.32	91.4	8.6	84.3	15.7	65	35	69.6	30.36	41.5	58.5	64.3	35.7	49.1	50.9	55.6	44.4
PORCEN	92.82513664				7.174863362				77.57759238				22.42240762				52.61019191				47.38980809			
	92.8	1.19							77.6	12.3							52.6	9.67						
				468								394								473				

Anexo 6. Continuación del anexo 5.

	3 mg/mL																															
	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
1	12	6	5	6	9	11	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
2	6	13	7	4	8	2	6	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
3	5	2	4	5	3	4	2	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
4	8	4	3	3	4	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
5	6	6	4	10	4	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
6	0	6	2	15	5	6	5	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
7	5	5	7	3	7	1	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
8	9	6	6	6	5	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
9	4	4	2	6	6	7	6	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
10	14	6	9	3	3	15	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
TOTAL	69	58	49	61	54	57	39	59	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
SUMA V	127		110		111		98		0		0		0		0		0		0		0		0									
PORCEN	54.33	45.7	44.5	55.5	48.6	51.4	39.8	60.2	####	####	####	####	####	####	####	#####	####	####	####	####	####	####	####	####								
PORCEN	46.83018256				53.16981744				#¡DIV/0!				#¡DIV/0!				#¡DIV/0!				#¡DIV/0!											
	46.8	6.17							####	####							####	####														
				446								0								0												

Anexo 7. Continuación del anexo 5 y 6.

Datos de julio Cruz Arévalo

FECHA DE LECTURA: 08/07/2016, 14:00 h inicio: 05/07/2016 14:00h

HORAS DE LECTURA: 72

	L3 SV vs Fracción 5 de *P. eryngii* 1255 en MeOH 4% acuoso (F5)																							
	MeOH 4%								IVM 5 mg/mL								F5 3 mg/mL							
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1	0	11	0	16	0	10	0	33	16	0	21	0	12	0	6	0	0	14	0	12	0	10	0	9
2	0	8	0	15	0	13	0	4	10	0	6	0	15	0	13	0	0	15	0	14	0	13	0	10
3	0	9	0	12	0	18	0	16	15	0	16	0	16	0	7	0	0	12	0	12	0	13	0	13
4	0	20	0	16	0	8	0	19	8	0	14	0	13	0	6	0	0	16	0	7	0	15	0	10
5	0	8	0	5	0	15	0	6	10	0	7	0	8	0	11	0	0	8	0	9	0	10	0	11
6	0	14	0	3	0	6	0	9	4	0	8	0	2	0	2	0	0	4	0	10	0	16	0	7
7	0	10	0	11	0	7	0	12	5	0	10	0	6	0	5	0	0	5	0	10	0	10	0	13
8	0	10	0	14	0	15	0	6	9	0	3	0	6	0	9	0	0	14	0	12	0	12	0	15
9	0	15	0	17	0	15	0	10	3	0	9	0	9	0	7	0	0	13	0	8	0	18	0	16
10	0	12	0	25	0	10	0	6	7	0	12	0	11	0	8	0	0	7	0	9	0	10	0	12
TOTAL	0	117	0	134	0	117	0	121	87	0	106	0	98	0	74	0	0	108	0	103	0	127	0	116
SUMA V	117		134		117		121		87		106		98		74		108		103		127		116	
PORCEN	0	100	0	100	0	100	0	100	100	0	100	0	100	0	100	0	0	100	0	100	0	100	0	100
PORCEN	0				100				100				0				0				100			
PROM Y	0	0							100	0							0	0						
				489	INDIVIDUOS MUESTREADOS							365								454				

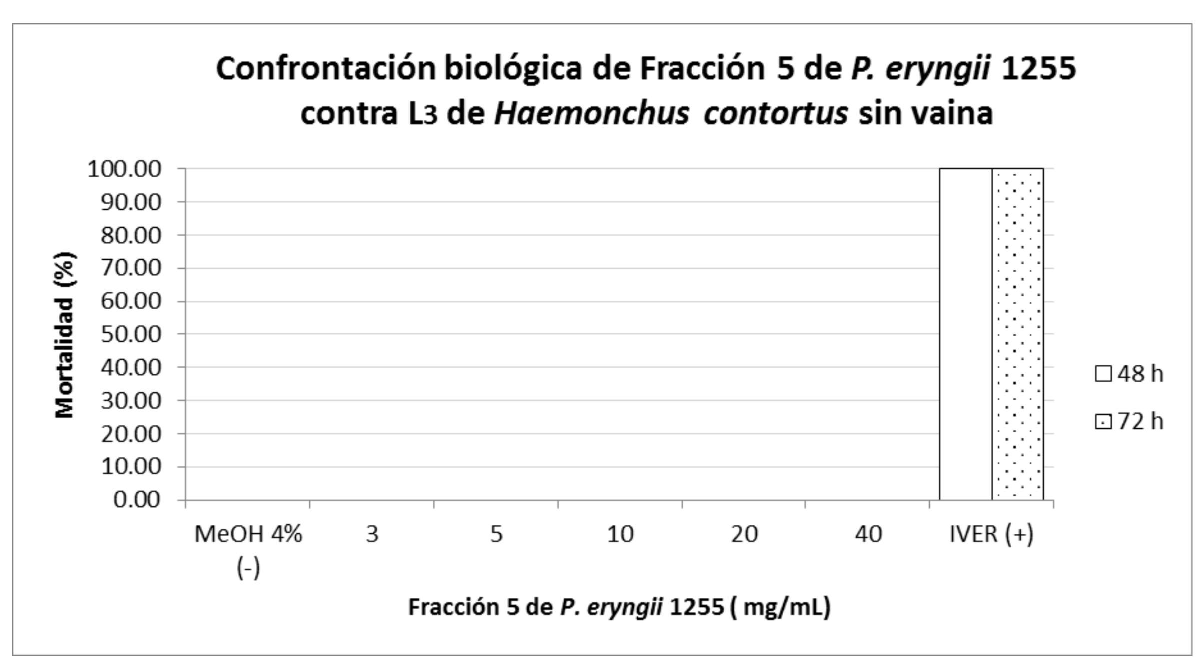
Anexo 8. Bioensayo fracción F5 contra larvas L3 sin vaina

	F5 5 mg/mL								F5 10 mg/mL								F5 20 mg/mL							
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1	0	17	0	9	0	14	0	13	0	5	0	8	0	8	0	14	0	12	0	17	0	9	0	8
2	0	8	0	15	0	8	0	15	0	11	0	9	0	8	0	4	0	9	0	17	0	13	0	14
3	0	11	0	20	0	5	0	9	0	9	0	18	0	14	0	13	0	3	0	17	0	11	0	19
4	0	10	0	10	0	11	0	7	0	7	0	14	0	7	0	8	0	11	0	12	0	11	0	16
5	0	6	0	9	0	8	0	13	0	9	0	15	0	14	0	9	0	12	0	13	0	12	0	12
6	0	4	0	11	0	6	0	4	0	7	0	9	0	5	0	6	0	8	0	10	0	11	0	4
7	0	9	0	5	0	8	0	7	0	13	0	3	0	8	0	8	0	11	0	6	0	11	0	8
8	0	10	0	4	0	11	0	8	0	8	0	10	0	14	0	5	0	7	0	6	0	10	0	4
9	0	14	0	14	0	13	0	10	0	10	0	7	0	10	0	12	0	13	0	13	0	7	0	6
10	0	16	0	10	0	7	0	7	0	13	0	11	0	8	0	12	0	8	0	7	0	8	0	12
TOTAL	0	105	0	107	0	91	0	93	0	92	0	104	0	96	0	91	0	94	0	118	0	103	0	103
SUMA V	105		107		91		93		92		104		96		91		94		118		103		103	
PORCEN	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
PORCEN	0				100				0				100				0				100			
	0	0							0	0							0	0						
				396								383								418				

Anexo 9. Continuación del anexo 8

	F5 40 mg/mL																							
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1	0	15	0	10	0	12	0	12																
2	0	23	0	16	0	12	0	4																
3	0	9	0	10	0	14	0	15																
4	0	15	0	7	0	13	0	9																
5	0	13	0	15	0	7	0	9																
6	0	8	0	3	0	11	0	9																
7	0	8	0	7	0	6	0	9																
8	0	8	0	6	0	11	0	6																
9	0	16	0	7	0	13	0	14																
10	0	12	0	15	0	10	0	13																
TOTAL	0	127	0	96	0	109	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SUMA V	127		96		109		100		0		0		0		0		0		0		0		0	
PORCEN	0	100	0	100	0	100	0	100	####	####	####	####	####	####	####	####	####	####	####	####	####	####	####	####
PORCEN	0				100				#¡DIV/0!				#¡DIV/0!				#¡DIV/0!				#¡DIV/0!			
	0	0							####	####							####	####						
				432								0								0				

Anexo 10. Continuación del anexo 9 y 10.



Anexo 11. Resultado de la confrontación de L_3 contra fracción F5

FECHA DE LECTURA: 23/06/2016, 11:00 h inicio: 20/06/2016 13:00 h

HORAS DE LECTURA: 72

L3 SV vs Extractos de P. eryngii MeOH 4% acuoso (FME)

	MeOH 4%										IV 25 mg/mL										ECS-1255 20 mg/mL									
	M	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**	**M**	**V**
1	0	30	0	11	0	12	0	3	0	39	5	0	22	0	7	0	5	0	1	0	2	8	2	8	2	8	2	8	2	10
2	0	4	0	8	0	26	0	15	0	1	6	0	12	0	8	0	10	0	15	0	0	7	5	7	2	4	2	8	1	8
3	0	6	0	9	0	7	0	12	0	4	3	0	17	0	2	0	12	0	4	0	2	8	1	4	1	7	2	18	1	12
4	0	2	0	2	0	4	0	12	0	0	4	0	12	0	8	0	3	0	3	0	2	6	0	3	1	7	2	13	1	3
5	0	3	0	12	0	3	0	11	0	2	6	0	16	0	4	0	5	0	3	0	2	3	1	7	0	7	0	0	1	1
6	0	3	0	0	0	0	0	3	0	7	0	0	3	0	3	0	2	0	1	0	0	8	2	5	1	4	0	2	1	4
7	0	1	0	3	0	2	0	3	0	14	1	0	2	0	2	0	10	0	3	0	0	0	1	3	1	5	0	0	2	7
8	0	1	0	5	0	0	0	0	0	0	0	0	9	0	5	0	5	0	4	0	0	0	0	7	2	2	0	0	2	6
9	0	1	0	2	0	3	0	6	0	1	3	0	7	0	3	0	9	0	2	0	0	0	0	0	2	2	0	0	2	4
10	0	0	0	9	0	5	0	3	0	0	4	0	9	0	10	0	5	0	6	0	0	0	0	0	2	2	0	0	1	3
TOTAL	0	51	0	61	0	62	0	68	0	68	32	0	109	0	52	0	66	0	42	0	8	40	12	44	14	48	8	49	14	58
SUMA V	51		61		62		68		68		32		109		52		66		42		48		56		62		57		72	
PORCEN	0	100	0	100	0	100	0	100	0	100	100	0	100	0	100	0	100	0	100	0	16.7	83.3	21.4	78.6	22.6	77.4	14	86	19.4	80.6
PORCEN	0				100						100				0						18.83108308				81.16891692					
PROM Y	0	0									100	0									18.8	3.5								
				242	INDIVIDUOS MUESTREADOS									259										223						

Anexo 12. Bioensayo de extractos hidroalcohólicos contra larvas L3

	ECS-1292 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	2	7		7	0	9	0	6	3	8
2	2	9	0	9	2	8	2	5	1	8
3	0	12	1	11	1	7	1	5	0	2
4	1	10	1	12	0	3	0	4	0	3
5	1	5	1	7	1	2	3	10	0	8
6	0	2	0	5	0	2	3	4	4	4
7	1	7	2	10	1	7	0	1	1	4
8	2	3	1	12	0	4	0	1	0	3
9	2	8	0	1	1	5	2	8	1	8
10	2	7	0	12	0	10	1	7	1	4
TOTAL	13	70	6	86	6	57	12	51	11	52
SUMA V	83		92		63		63		63	
PORCEN	15.7	84.3	6.52	93.5	9.52	90.5	19	81	17.5	82.5
PORCEN	13.64322715				86.35677285					
	13.6	5.37								
				364						

	ECS-1138 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	3	12	2	14	0	11	0	5	3	15
2	2	8	0	5	3	5	6	22	1	8
3	1	10	2	10	3	14	2	11	1	14
4	2	11	3	11	1	4	0	7	0	8
5	1	7	1	12	0	5	1	2	0	5
6	0	0	1	2	1	1	0	1	0	4
7	0	1	0	5	0	5	0	0	0	0
8	0	6	0	7	1	3	0	2	0	0
9	0	6	1	6	1	9	0	1	0	0
10	1	9	1	13	0	0	0	0	0	0
TOTAL	10	70	11	85	10	57	9	51	5	54
SUMA V	80		96		67		60		59	
PORCEN	12.5	87.5	11.5	88.5	14.9	85.1	15	85	8.475	91.53
PORCEN	12.47165655				86.52907338					
	12.5	2.71								
				362						

	ECS-1258 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	1	6	6	16	3	6	12	68	1	8
2	2	7	1	1	7	22	4	19	0	6
3	0	4	0	1	1	6	1	16	1	7
4	2	10	1	8	1	2	0	3	2	6
5	1	9	1	0	0	4	1	6	3	9
6	0	4	0	0	0	0	0	0	0	0
7	3	28	0	0	0	2	0	3	2	4
8	0	0	0	0	0	0	1	7	0	7
9	0	0	0	2	0	1	1	2	0	2
10	0	0	1	2	1	2	0	0	1	6
TOTAL	9	68	10	30	13	45	20	124	10	55
SUMA V	77		40		58		144		65	
PORCEN	11.7	88.3	25	75	22.4	77.6	13.9	86.1	15.4	84.6
PORCEN	17.67512181				82.32487819					
	17.7	5.73								
				384						

Anexo 13. Continuación del anexo 12

	ECS-1282 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	1	12	2	8	0	2	1	9	0	0
2	1	11	1	11	0	4	3	15	0	0
3	1	12	1	9	2	6	3	5	1	0
4	0	8	2	7	0	4	1	13	1	0
5	0	5	2	4	0	8	0	11	3	0
6	0	2	1	0	0	13	0	1	1	4
7	0	2	0	0	1	12	0	2	0	0
8	0	2	1	4	2	6	0	4	1	1
9	0	9	0	2	0	9	1	0	0	36
10	0	0	0	0	0	5	1	3	0	0
TOTAL	3	63	10	45	5	69	10	63	7	41
SUMA V	66		55		74		73		48	
PORCEN	4.55	95.5	18.2	81.8	6.76	93.2	13.7	86.3	14.6	85.4
PORCEN	11.55319859				88.44680141					
	11.6	5.7								
				316						

	ECS-1156 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	2	14	1	8	1	8	1	4	1	7
2	3	16	0	11	0	6	1	3	2	12
3	0	8	1	7	4	5	0	3	0	9
4	0	8	3	8	4	1	1	8	3	9
5	2	5	0	10	3	3	1	8	0	7
6	0	5	0	8	0	1	0	10	0	0
7	4	9	2	8	2	7	1	13	0	2
8	0	9	1	7	0	8	0	5	0	2
9	1	8	3	4	0	2	3	10	4	4
10	0	0	0	12	1	5	0	0	2	6
TOTAL	12	82	11	83	15	46	8	64	12	58
SUMA V	94		94		61		72		70	
PORCEN	12.8	87.2	11.7	88.3	24.6	75.4	11.1	88.89	17.14	82.86
PORCEN	15.46244346				84.53755654					
	15	5.62								
				391						

	ECS-1261 20 mg/mL									
	M	V	M	V	M	V	M	V	M	V
1	2	16	1	9	0	50	3	7	0	13
2	2	16	0	16	1	19	1	4	0	8
3	1	2	0	5	3	17	2	2	0	4
4	1	3	1	4	0	12	3	4	1	5
5	1	1	0	0	3	12	1	2	1	4
6	1	5	0	1	2	8	0	0	0	0
7	2	7	1	4	0	9	0	2	0	2
8	0	10	0	0	2	17	1	5	1	0
9	0	2	1	5	0	7	0	7	1	1
10	0	0	0	0	0	3	2	5	0	4
TOTAL	10	62	4	44	11	154	13	38	4	41
SUMA V	72		48		165		51		45	
PORCEN	13.9	86.1	8.33	91.7	6.67	93.3	25.5	74.5	8.89	91.1
PORCEN	12.65359477				86.40522876					
	12.7	7.66								
				381						

Anexo 14. Continuación del anexo 12 y 13

FECHA DE LECTURA: 03/NOV/2016

HORAS DE LECTURA: 72

inicio: 14:00 h

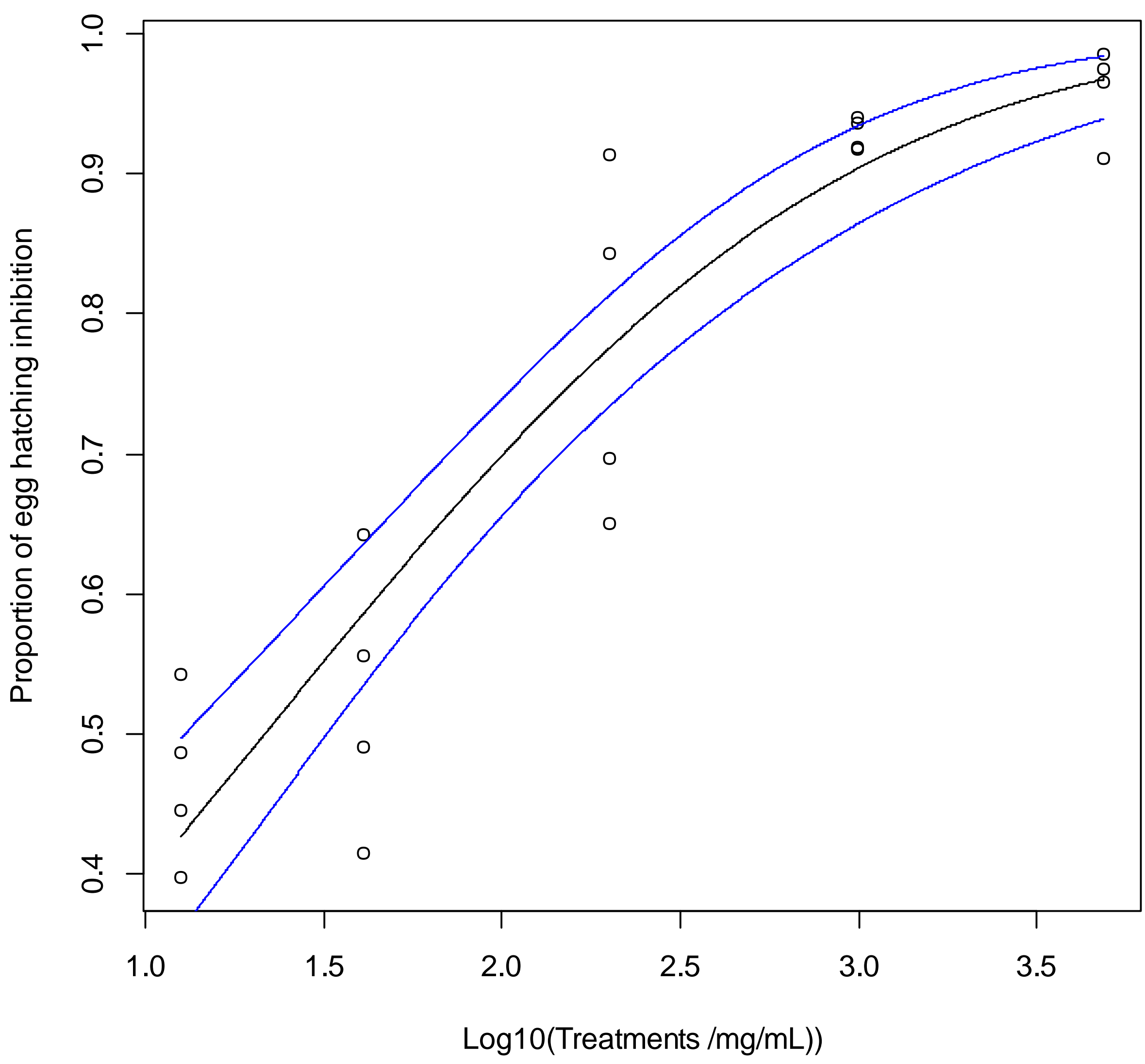
Pruebas tween-80, DMSO 2.5 % y MeOH 4 % en PBS

	PBS + Tween-80								PBS + Tween-80 + DMSO								PBS + tween-80 + MeOH								Tween-80 + PBS + escualeno							
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1		16		8		17		7		15		11		14		9		28		4		5		6		0		1		6		6
2		6		8		3		10		11		21		14		5		7		4		5		5		1		3		4		5
3		2		12		7		8		15		13		11		9		9		5		6		12		7		6		2		5
4		5		3		6		6		12		10		22		13		3		4		7		11		8		3		9		8
5		7		7		8		10		15		18		11		10		7		15		14		13		4		4		4		2
6		2		5		4		10		4		7		1		5		4		3		6		7		3		1		6		2
7		6		8		6		10		10		5		10		8		4		1		3		10		3		5		9		9
8		17		9		7		5		16		7		9		10		4		5		7		3		4		7		5		7
9		7		3		2		9		10		21		8		7				6		11		12		1		4		8		4
10		10		17		7		21		9		16		13		12				7		18		4		2		4		10		6

	Tween + PBS + DMSO + escualeno								Tween + MeOH + PBS + escualeno								Tween + PBS + Boldo								Tween + PBS + DMSO + Boldo							
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1		2		10		8		10		21		11		12		21	8		20		16		15		10		29		11		12	
2		6		12		5		5		6		7		12		4	17		11		13		4		7		43		10		15	
3		4		5		4		7		7		6		13		10	16		14		8		10		6		24		12		13	
4		10		15		11		3		7		4		8		4	7		11		6		12		3		38		9		6	
5		15		5		4		7		6		6		10		7	8		7		8		6		16		37		10		10	
6		1		0		1		6		10		5		2		1	4		4		0		5		9		4		9		3	
7		0		3		3		2		0		0		0		0	6		8		8		4		10		36		7		11	
8		7		0		5		9		4		1		4		2	9		13		15		9		6		39		8		9	
9		6		3		5		4		4		10		2		0	6		10		10		8		8		27		8		11	
10		5		2		5		7		4		2		7		2	14		9				6		10							

	Tween + PBS + MeOH + ~~MeO~~ Boldo																															
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
1	6		8		9		14																									
2	8		11		10		10																									
3	10		8		10		6																									
4	8		8		10		5																									
5	4		15		9		8																									
6	13		1		6		7																									
7	5		4		8		10																									
8	8		11		7		9																									
9	5		10		9		11																									
10	12				5		9																									

Anexo 15. Datos crudos de la actividad nematicida del escualeno contra larvas L_3



Anexo 16. Gráfico del análisis Probit de la confrontación huevo vs fracción F5 ajustado a un modelo lineal generalizado

"2015, Año del Generalísimo José María Morelos y Pavón"

Jiutepec, Morelos, diciembre 04 de 2015.

POSGRADO UNIDAD TAPACHULA
COLEGIO DE LA FRONTERA SUR,
UNIDAD TAPACHULA, CHIAPAS

Sirva la presente para informar que el bienestar de los animales y el sufrimiento innecesario de los animales son políticas de Buenas Prácticas de Manejo bien establecidas en nuestra institución. Por esta razón, y para asegurar el bienestar animal cuando se incluyen animales de experimentación en los trabajos de investigación realizados en el Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria (CENID-PAVET) del Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), nos atenemos a la Norma Oficial Mexicana NORMA Oficial Mexicana NOM-051-ZOO-1995, Trato humanitario en la movilización de animales (http://www.dof.gob.mx/nota_detalle.php?codigo=4870842&fecha=23/03/1998), así como la LEY FEDERAL de SANIDAD ANIMAL (http://www.diputados.gob.mx/LeyesBiblio/pdf/LFSA.pdf), documentos que mencionan que todos los procedimientos realizados en estudios con animales deben estar en conformidad con los reglamentos y lineamientos normativos existentes en el País y, en nuestro caso particular, con las normas de ética establecidas en el INIFAP.

El protocolo de investigación intitulado **"Identificación del compuesto activo anti-helmíntico de *Pleurotus eryngii* aplicado como desparasitante de ovinos, *Haemonchus contortus*"** cuyo sustentante es el estudiante **Julio Cruz Arévalo,** cumplirá con los requisitos mínimos esenciales en cuanto a manejo de animales en los experimentos que habrán de realizarse en este CENID-PAVET, manteniendo la idoneidad de los elevados estándares de bienestar y salud animal.

Sin otra por el momento, hago propicia la ocasión para enviarle un cordial saludo.

ATENTAMENTE
DIRECTOR DEL CENID-PAVET

CENID PAVET

DR. JULIO VICENTE FIGUEROA MILLÁN

C.c.p. Dr. José Ernesto Sánchez Vázquez.- Posgrado Unidad Tapachula, Colegio De La Frontera Sur, Unidad Tapachula, Chiapas.
Dra. Liliana Aguilar Marcelino.- Investigador Titular C en el CENID-PAVET.
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