

Short Note [Nota corta]



EVALUACIÓN *in vitro* DE EXTRACTOS DE HONGOS COMESTIBLES EN LA PRUEBA DEL DESENVAINE LARVAL CONTRA *Haemonchus contortus*[†]

[*In vitro* ASSESSMENT OF EDIBLE MUSHROOM EXTRACTS IN THE LARVAL EXSHEATHMENT TEST AGAINST *Haemonchus contortus*]

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SUMMARY

Background. Worldwide, gastrointestinal nematodes (GIN) cause losses in livestock production, because in some animals they can cause weight loss or death. Combating these GIN has been based on the use of anthelmintics. However, the misuse of these treatments has caused anthelmintic resistance. Therefore, there is currently a search for new biological alternatives for the control of gastrointestinal nematodes. One of these alternatives is the use of fungal extracts for nematode control. **Objectives.** To utilize the *in vitro* larval exsheathment inhibition test to evaluate organic extracts of edible fungi *Pleurotus eryngii*, *P. djamor* and *Lentinula edodes*, against *Haemonchus contortus* (L3). **Methodology.** Extracts were prepared from the basidiomes of the fungal species mentioned. In the case of *P. eryngii* and *P. djamor*, they were placed in a hydroalcoholic mixture (methanol/water 70:30). On the other hand, *L. edodes* basidiomes were macerated with distilled water for 24 hours. The extracts were filtered with a cotton/gauze system and through Whatman paper (#4) and were concentrated using a rotary evaporator until the liquid residue was removed and kept at -4 °C until use. To determine the percentage of larval exsheathment inhibition, larvae were exposed to different concentrations (156.25, 312.5, 625, 1250, and 2500 µg/mL) with their respective negative controls (PBS) for 60 minutes. The effective concentration 50% (EC₅₀) was calculated by means of probit analysis. **Results.** The extracts with the best activity were *P. djamor* and *L. edodes* with an effective concentration (EC₅₀) of 533.3 and 558.5 µg/mL, respectively. **Implications.** This *in vitro* evaluation provides results that suggest the need for further *in vitro* studies with more fungi species and other types of extraction procedures. **Conclusion.** The present study demonstrated that the use of extracts of *P. eryngii*, *P. djamor* and *L. edodes* was shown to have *in vitro* anthelmintic activity against the larval exsheathment of *H. contortus*.

Keywords: exsheathment test; *Haemonchus contortus*; basidiomata; *Pleurotus*; *Lentinula edodes*.

RESUMEN

Antecedentes. A nivel mundial los nematodos gastrointestinales (NGI) causan pérdidas en la producción ganadera, debido a que en algunos animales pueden llegar a ocasionar pérdida de peso o la muerte. El combate a estos NGI se ha basado en el uso de antihelmínticos. Sin embargo, el mal uso de estos tratamientos ha provocado resistencia antihelmíntica. Por lo tanto, actualmente existe la búsqueda de nuevas alternativas biológicas para el control de nematodos gastrointestinales. Una de estas alternativas es el uso de extractos de origen fúngico para el control de nematodos. **Objetivos.** Utilizar la prueba *in vitro* de inhibición de desenvaine de larvas para evaluar los extractos orgánicos de los hongos comestibles *Pleurotus eryngii*, *P. djamor* y *Lentinula edodes*, contra *Haemonchus contortus* (L3). **Métodos.** Los extractos fueron elaborados a partir de los basidiomas de las especies de hongos mencionadas.

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Para el caso de *P. eryngii*, y *P. djamor* se colocaron en una mezcla hidroalcohólica (metanol/agua 70:30). Por otra parte, los basidiomas de *L. edodes* se maceraron con agua destilada, durante 24 horas. Los extractos se filtraron con un sistema de algodón/gasa y a través de papel Whatman (#4) y se concentraron usando un rotavapor, hasta que se eliminó el residuo líquido y se mantuvieron a -4°C hasta su uso. Para determinar el porcentaje de inhibición del desenvaine, las larvas fueron expuestas a diferentes concentraciones (156.25, 312.5, 625, 1250 y 2500 $\mu\text{g/mL}$) del extracto con sus respectivos controles negativos (PBS) durante 60 minutos. Los resultados fueron analizados y se calculó la concentración eficaz 50% (CE_{50}) por medio de análisis probit. **Resultados.** Los extractos con la mejor actividad fueron *P. djamor* y *L. edodes* con una CE_{50} de 533.3 y 558.5 $\mu\text{g/mL}$, respectivamente. **Implicaciones.** Esta evaluación *in vitro* aporta resultados que sugieren la necesidad de realizar más estudios *in vitro* con más especies de hongos y otros tipos de procedimientos de extracción. **Conclusiones.** El presente estudio demostró que el uso de extractos de *P. eryngii*, *P. djamor* y *L. edodes* poseen actividad antihelmíntica *in vitro* ya que inhiben el desenvaine de larvas de *H. contortus*. **Palabras clave:** Test de desenvaine; *Haemonchus contortus*; basidiomas; *Pleurotus*; *Lentinula edodes*.

INTRODUCTION

Gastrointestinal nematodes (GIN) are among the main parasites affecting ruminants, particularly sheep and goats (Thumbi *et al.*, 2014). *Haemonchus contortus* is considered one of the most important GIN as this agent can cause severe clinical signs in small ruminants (López-Arellano *et al.*, 2010). These parasites have been controlled through the use of synthetic anthelmintic products, being the most effective control method established worldwide (Braga & Araujo, 2014, Jackson & Miller, 2006). The most common anthelmintic drugs used for the control of GIN in ruminants are benzimidazoles, macrocyclic lactones, and imidazothiazoles. The latter are used without a medical prescription, resulting in extensive and irrational use leading to the development of anthelmintic resistance in the target parasites (López *et al.*, 2015). Anthelmintic resistance results from the development of one or more mutations in the parasitic population caused by the constant administration of these chemicals (Medina *et al.*, 2014). Other problems have been triggered by the inappropriate use of antiparasitic drugs against nematodes, including ecotoxicity in water sources and ecological niches, damaging non-target organisms including beneficial microorganisms such as dung beetles (*Podotenus fulviventris*, *Aphodius pseudolividus*, *Aphodius granarius*), earthworms, nematophagous mites (*Caloglyphus mycophagus*; Quintero-Elena *et al.*, 2022), and the water flea (*Daphnia magna*).

In view of this situation, comprehensive strategies for the control of GIN are being sought from a sustainable perspective that considers conservation and respect for the environment. Recent studies have proposed the use of edible mushrooms as GIN control alternative. The latter is based on evidence suggesting the diverse medicinal, therapeutic, and nutritional properties of different species of edible mushrooms, including their anti-parasitic action, particularly as nematicides (Aguilar-Marcelino *et al.*, 2017).

Several studies found that fungi can show antiparasitic activity against GIN. In this regard, *Pleurotus* spp.

produces secondary metabolites with nutraceutical, immunotherapeutic, antiparasitic, cytotoxic, antitumoral, and anticancer properties (Moradali *et al.*, 2007; Erjavec *et al.*, 2012; Hassan *et al.*, 2015). The active metabolites have mostly been isolated from fungal basidiomes (Aguilar-Marcelino *et al.*, 2017). One of the control alternatives is the use of fungal extracts that have shown anthelmintic effects. In addition, these mushrooms were selected because they are highly consumed in the world, for their properties and flavor. Therefore, it has a worldwide economic importance (Boa, 2004). The procedures used for extraction were different because we decided to use the extracts with the highest *in vitro* activity against larvae for the different types of edible mushroom species according to previous studies reported (Cruz-Areválo *et al.*, 2020; Pineda-Alegría, 2019; González-Cortázar *et al.*, 2021). Therefore, this study aims at evaluating the *in vitro* larval exsheathment of *H. contortus* L₃ caused by the organic extracts of the edible fungi *P. eryngii*, *Lentinula edodes*, and *P. djamor*.

MATERIALS AND METHODS

Experimental work was carried out at the Helminthology Laboratory of the National Centre for Disciplinary Research in Animal Health and Safety of the National Institute of Forestry, Agriculture and Livestock Research (CENID-SAI, INIFAP), Jiutepec, Morelos, Mexico.

Biological material

Infection and recovery of *Haemonchus contortus* infective larvae

A three-month-old male sheep (donor) weighing 20 ± 1 kg was infected orally with 350 larvae (L₃) of *H. contortus* per kg live weight. The donor sheep was kept under controlled feeding conditions and isolated in a metabolic cage. After 21 days, the presence of nematodes in the donor was confirmed by the McMaster technique. Once the presence of nematodes in the donor was confirmed, stool cultures were prepared from fecal samples obtained from the donor

sheep (MAFF, 1986). Stool cultures were incubated for 7 days, and infective *H. contortus* larvae were then obtained. These larvae were recovered using the Baermann funnel technique, washed with distilled water to remove fecal matter, and then stored at 6–8 °C until use.

Preparation of fungal extracts

Basidiomata samples from *P. eryngii* (CEPA 1292), *P. djamor* (CEPA 123- accessing number: GU722265), and *L. edodes* (CEPA 401) were obtained from ECOSUR, Tapachula, Chiapas, México. These edible mushroom species were selected because they are highly consumed in the world, for their properties and flavor. Therefore, it has a worldwide economic importance (Boa, 2004). The selected strains were chosen due to their high demand in Latin America and Mexico, since these are the fungi species with the highest production and consumption, as well as their easy cultivation under controlled conditions and domestication. The selection of these edible mushrooms was also based on the previous results of anthelmintic activity (Cruz-Areválo *et al.*, 2020; Pineda-Alegría *et al.*, 2021; González-Cortázar *et al.*, 2021). The samples of *P. eryngii* and *P. djamor* (2 kg) were macerated in a hydroalcoholic mixture (methanol/water 70:30). Meanwhile, the *L. edodes* sample (2 kg) was macerated with distilled water. The procedures used for extraction were different because previous studies reported higher *in vitro* activity against larvae for the different types of fungal extracts used (Cruz-Areválo *et al.*, 2020; Pineda-Alegría, 2019; González-Cortázar *et al.*, 2021). The amount of solvent used in relation to the sample size was a 1:3 ratio (fungal material: solvent). The fungal material was then incubated for 24 h at room temperature. The extracts were filtered with a cotton/gauze system through Whatman paper No. 4 and concentrated using a rotary evaporator until the liquid residue (methanol) was removed. The fungal material was macerated with distilled water (1:3 w/v) for 24 hours, then the material was filtered and concentrated in a rotary evaporator.

Larval exsheathment inhibition test (LEIT)

Tests were performed following the procedure described by Jackson and Hoste (2010). The extract concentrations used in the LEIT were adopted from the protocols described to test extracts from plant materials (Chan-Pérez *et al.*, 2017; Alonso-Díaz *et al.*, 2008; Castañeda-Ramírez *et al.*, 2018). An initial stock solution of 10,000 µg/mL of each extract in phosphate-buffered saline (PBS) was prepared. In plastic tubes, a one mL volume of the extracts was prepared at final serial concentrations of 156.25, 312.5, 625, 1250 and 2500 µg/mL and a tube with PBS was also included (negative control). One mL of the larval suspension (~1000 L₃/mL) was added to each tube. The tubes were

incubated for 3 h at 21 °C. During incubation, the tubes were shaken every hour. After incubation, washes (3×) were initiated for three minutes at 1500 rpm, removing one mL of the supernatant and adding one mL of PBS. The remaining 1 mL in each tube was transferred to 4 vials in 200 µL aliquots of the solution (4 replicates for each concentration tested). The vials were stored in the refrigerator (11 °C) for 12 h before testing. The chlorine concentration that induced larval exsheathment was determined from an extra series of PBS samples (control group). The chlorine concentrations used were 4.0–6.0% made from sodium hypochlorite and a solution of 0.002–0.10% was made from sodium hydroxide. Each concentration was analyzed for 0, 20, 40, and 60 minutes to determine the most effective concentration for exsheathment. This methodology was performed according to Chan-Pérez *et al.* (2017).

Evaluation of the exsheathment process

At minute 0, 50 µL were taken from each vial and placed on the slide, and then the coverslip was applied. After mounting the aliquots on the slides. Each concentration was counted, and at minute 0, the larvae still had their sheaths. Then, 150 µL of the chlorine solution (to induce larvae exsheathment) was added to the tubes. The number of larvae that were observed at each concentration during the different time intervals (20, 40, and 60 minutes) and the number of larvae that were sheathed and were not sheathed was recorded.

Data analysis

The exsheathment inhibition percentage (EI) at minute 60 was determined for each extract using the formula described by Chan-Pérez *et al.* (2017):

$$\text{Exsheathment \%} = \frac{(\text{larvae without sheath})}{(\text{larvae with sheath} + \text{larvae without sheath})} \times 100$$

$$\text{EI \%} = 100 - \text{Exsheathment}$$

An analysis of variance (ANOVA) was performed to analyze the data of exsheathment inhibition. The means at different concentrations were compared ($\alpha = 0.05$) using a mean comparison test with Statgraphics software. In addition, the effective concentrations (EC₅₀ and EC₉₀) were determined by Probit analysis (Polo plus-Leora Software).

RESULTS

Table 1 and Figure 1 show the EI percentage of *H. contortus* L₃ resulting from the incubation with hydroalcoholic extracts of *P. djamor*, *P. eryngii* and *L. edodes* fungi. A 100% exsheathment inhibition was recorded at a concentration of 2500 µg/mL *P. djamor*

and *L. edodes* extracts. These same fungal extracts showed EI activity that was significantly different from PBS at 625 µg/mL. Meanwhile, at the higher concentration tested (2500 µg/mL) the *P. eryngii* extract only showed an EI activity of 67.9%.

Table 1. Exsheathment inhibition percentage of *Haemonchus contortus* L₃ larvae after 60 minutes of exposure to different concentrations of organic extracts of *Pleurotus djamor*, *P. eryngii* and *Lentinula edodes*.

Concentration (µg/mL)	% Exsheathment inhibition		
	<i>Pleurotus djamor</i>	<i>P. eryngii</i>	<i>Lentinula edodes</i>
Phosphate	1.3 ^A	1.4 ^A	2.1 ^A
Buffer Saline			
156.3	2.6 ^A	1.9 ^A	7.1 ^A
312.5	8.2 ^A	4.9 ^A	5.9 ^A
625.0	60.4 ^B	11.7 ^A	56.2 ^B
1250.0	99.2 ^C	26.2 ^A	100.0 ^C
2500.0	100.0 ^C	67.9 ^B	100.0 ^C
Standard Error	4.42	7.9	6.5

A, B, C represent significant differences ($p < 0.05$) within row compared to the PBS control.

Table 2 shows the effective concentrations 50% and 90% (EC₅₀ and EC₉₀) of *P. djamor*, *P. eryngii*, and *L. edodes* extracts against *H. contortus* L₃ larvae exsheathment. The extracts with the best activity were *P. djamor* and *L. edodes* (EC₅₀: 533.3 and 558.5 µg/mL, respectively).

Figure 2 shows the exsheathment process of *H. contortus* infective larvae, including the start of exsheathment (Figure 2a) and the moment when L₃ larvae detach from their sheath (Figure 2b). An L₃ showing exsheathment inhibition is also shown (Figure 2c).

DISCUSSION

The *in vitro* activity of *P. eryngii*, *P. djamor* and *L. edodes* against *H. contortus* L₃ exsheathment was evaluated. This study focused on measuring the ability of the different extracts to inhibit the exsheathment of L₃ larvae. This test has been associated with the disruption of the normal life cycle of L₃ by exposure of these larvae to extracts of fungal or plant materials (Brunet *et al.*, 2008). To the best of our knowledge, this is the first study to evaluate the activity of extracts from these different edible mushroom species using this *in vitro* test (LEIT).

In comparison to other fungal studies and other *in vitro* tests, with respect to the fungus *P. eryngii*, Comans-

Perez *et al.* (2021) reported that the mycelial extract caused 95% mortality in infective *H. contortus* larvae after 48 h of exposure *in vitro*.

The *P. djamor* has many potential biotechnological applications. Pineda-Algeria *et al.* (2017) reported anthelmintic activity of the hydroalcoholic extracts against *H. contortus*. These authors evaluated the extract and its fractions in the *in vitro* mortality test against larvae and concluded that *P. djamor* can be considered as a potential alternative to reduce the use of conventional anthelmintic treatments due to different compounds present in the active fraction, such as fatty acids (pentadecanoic, hexadecenoic, octadecadienoic and octadecanoic acids). Meanwhile, González-Cortázar, *et al.* (2021) identified, isolated and evaluated the metabolites responsible for the nematocidal activity of the fractions from the hydroalcoholic extract of *P. djamor*, reporting that the fraction called “PdB” showed 100% inhibition of the hatching of eggs of *H. contortus*. The PdB fraction with anthelmintic activity against *H. contortus* contained allitol and a terpene that remains to be identified and evaluated separately as the main components.

On the other hand, the *L. edodes* edible mushroom is known for the presence of bioactive compounds such as palmitic acid, linoleic acid, lentinan and ergosterol and has also been reported to be high in protein content (22.8%) and fiber. Its anthelmintic activity against *H. contortus* larvae has been reported with different larvae mortality tests (Pineda-Alegría, 2019). That study reported a different test in L₃ larvae, and the activity was observed 68% of mortality at higher concentrations (100mg/mL).

However, in spite of the studies reported above, they cannot be fully comparable, due to the fact that the larval exsheathment inhibition test has shown to be more sensitive compared to other *in vitro* methodologies (Alonso-Díaz *et al.*, 2008). Therefore, it is necessary to continue evaluating with this methodology (LEIT), in order to compare the results in future studies.

Thus, the fungi that showed the highest percentage of exsheathment inhibition were *P. djamor* and *L. edodes*. However, the type of extraction used for these two fungi materials was different. In the present study, the selection of the extraction procedure was based on previous studies where activity of the extracts against larval mortality was reported (Pineda-Alegría, 2019; González-Cortázar *et al.*, 2021; Cruz-Arévalo *et al.*, 2020). On the other hand, these extraction variations may show different active compounds.

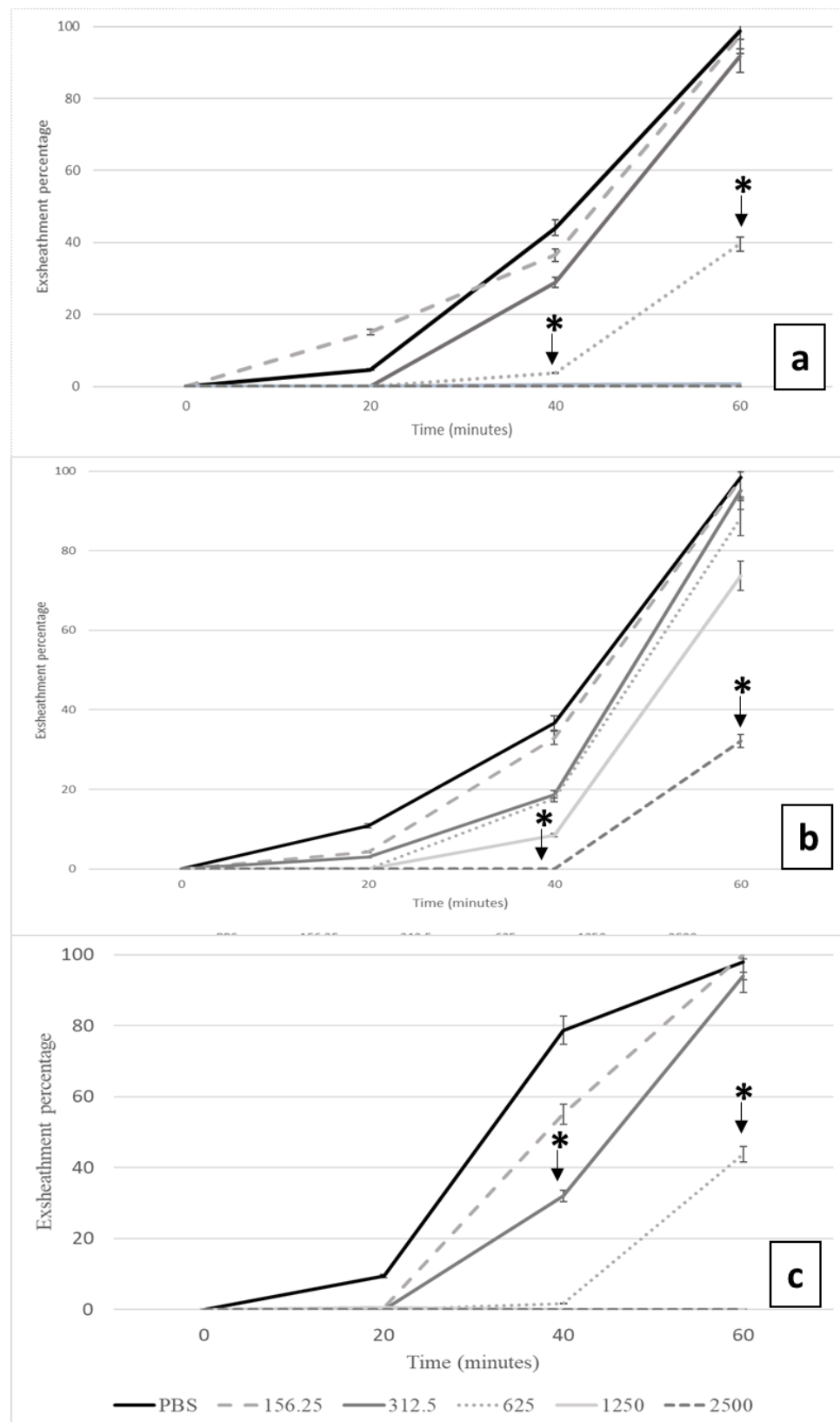


Figure 1. Exsheathment percentage of *Haemonchus contortus* L₃ incubated for a 60-minute period in different extract concentrations (from 156.25 to 2500 µg/mL). Extracts were prepared with basidiomata from edible mushrooms: (a) *Pleurotus djamor*, (b) *Pleurotus eryngii*, and (c) *Lentinula edodes*. The arrow points the concentrations with significant differences compared to the negative controls (PBS) at 40 and 60 minutes.

Table 2. Effective concentrations 50% (EC₅₀) and 90% (EC₉₀) of *Pleurotus djamor*, *P. eryngii*, and *Lentinula edodes* extracts against *H. contortus* L₃ larvae exsheathment.

Extracts	EC ₅₀ (µg/mL)	Confidence interval	EC ₉₀ (µg/mL)	Confidence Interval
<i>Pleurotus. djamor</i>	533.3 ^A	441.4- 652.9	949.8 ^A	755.7- 1412. 8
<i>P. eryngii</i>	1987.0 ^B	1447.3- 4052.1	-	-
<i>Lentinula edodes</i>	558.5 ^A	494.7- 631.4	856.7 ^A	740.2- 1086.3

^{A, B, C} represent significant differences ($p < 0.05$) within columns. – The concentrations used for the *P. eryngii* extract were insufficient to estimate the EC₉₀.

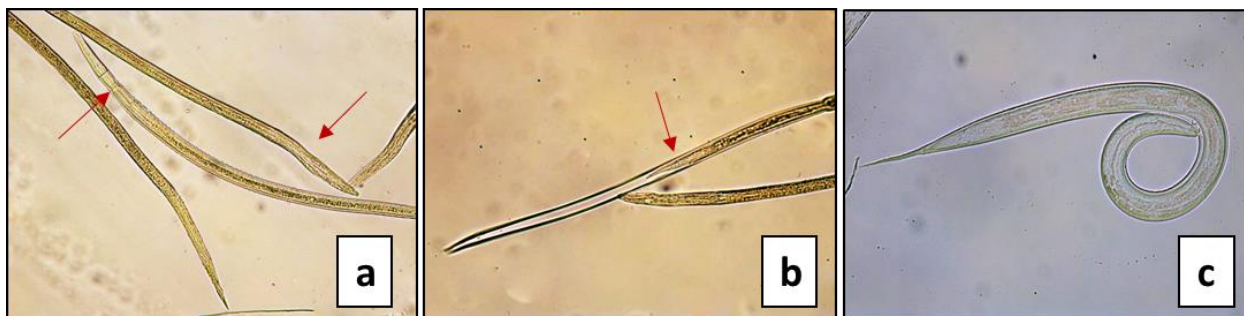


Figure 2. Overview of the effect of organic extracts of *Pleurotus djamor*, *P. eryngii*, and *Lentinula edodes* on the exsheathment of *Haemonchus contortus* infective larvae: (a) L₃ larvae beginning the ecdysis process showing the shrinkage of the sheath in the anterior section; (b) an infective larva in the process of losing its sheath, showing the posterior section of the larva tail emerging from its sheath; and (c) infective larva with inhibited exsheathment.

The water extract of *L. edodes* was as active as *P. djamor* extract even though the latter was obtained with methanol:water (70:30). It is well known that water is used to extract the polar compounds, while the methanol:water extracts can also extract polar compounds, as well as a variety of compounds with less polarity. Both types of solvents can be used to extract polyphenols, phenolic acids, flavonoids glycosides (carbohydrates, saponins) and some polypeptides. Thus, it is not possible to know whether the EI activity was associated with compounds present in both types of extracts or was related to compounds that were exclusive of each extract. Respective bioguided fractionation with microchemical studies are necessary to identify the compounds associated with the activity in each of the extracts. Previous studies have confirmed that extracts of different polarity can induce an EI activity in *H. contortus* L₃, and these authors also proposed the possible role of different compounds on the reported activity (Hernández-Bolio, 2017). For the case of *P. djamor* (ethanol-96%), different compounds have already been reported in active fractions, pentadecanoic, hexadecanoic, octadecadienoic, octadecanoic acid, allitol and β -sitosterol (Pineda-Alegría *et al.* 2017; Gonzalez-Cortazar *et al.*, 2021). Compounds have also been reported in active fractions for *P. eryngii* including the

compounds Trehalose, D-glucitol, myoinositol, adipic acid, squalene, stearic acid and β -sitosterol (Cruz-Arevalo *et al.* 2020).

For the fungus *L. edodes* fractions with anthelmintic activity, the compounds found were mainly terpenes, flavonoids and linoleic acid (Pineda-Algeria, 2019; Jang *et al.* 2015). Similarly, previous studies have attributed anthelmintic activity to the toxin trans-2-decenoic acid, which is present in this fungus and it seems responsible for the inhibition of nematode movements and subsequently cause their degradation (Kwok *et al.* 1992). These same previous studies have reported the importance of mixtures of secondary compounds in the active fractions and their importance in the anthelmintic activity. In addition, no single molecule has yet been attributed activity against nematodes by these fungi. With the possible metabolites previously reported from these types of extracts, it could be possible that some of the compounds block enzyme channels. A 44KD zinc metalloproteinase responsible for the digestion of the sheath (L₃), among other mechanisms involved in the exsheathment process, has been observed to be involved (Gamble *et al.* 1989). More studies are still needed to clearly and concisely identify the mechanisms of action in the unsheathing process with respect to the different compounds in the extracts evaluated.

CONCLUSION

Based on the *in vitro* evaluation of the exsheathment test of *P. eryngii*, *P. djamor*, and *L. edodes* fungal extracts against *H. contortus* (L3), we concluded that *P. djamor* and *L. edodes* extracts can inhibit the exsheathment by 100% from a concentration of 1250 µg/mL. Meanwhile, the *P. eryngii* extract was less effective, with only 68% inhibition at the highest concentration tested. Based on the EC₅₀ values, the organic extracts of *P. djamor* (533.3 µg/mL) and *L. edodes* (558.5 µg/mL) were determined to be the most effective. This *in vitro* evaluation provides results that suggest the need for further *in vitro* studies to identify the compounds involved in the activity and their mechanism of action.

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Conflict of interest. None.

Compliance with ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant Mexican guidelines regarding animal welfare and unnecessary animal suffering; these are Good Management Practices policies well established at our institution. The Norma Oficial Mexicana (Official Rule Number) NOM-051-ZOO-1995 (<http://www.senasica.gob.mx>) as well as the Ley Federal de Sanidad Animal (Federal Law for Animal Health) DOF 07-06-2012 (<http://www.diputados.gob.mx/LeyesBiblio/ref/lfsa.htm>) were strictly abided to and all the procedures performed in studies involving animals were in accordance with the ethical standards at INIFAP.

Data availability. Data are available under request with Dr. Liliana Aguilar-Marcelino (aguilar.liliana@inifap.gob.mx), and Dr. Gloria Sarahi Castañeda-Ramírez, (gs.castanedaramirez@gmail.com).

Author contribution statement. **J. Ambrosio-Bautista** – Methodology, writing, review & editing., **M. Rodríguez-Labastida** – Writing, review & editing., **J.E. Sanchez-Vázquez** - Writing, review & editing., **J.F.J. Torres-Acosta** – Writing, review & editing., **G.S. Castañeda-Ramírez** - Methodology, Supervision, Writing, review & editing, and **L.**

Aguilar-Marcelino - Methodology, Supervision, Funding acquisition, Writing, review & editing.

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