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Assessing the *In Vitro* Individual and Combined Effect of *Arthrobotrys oligospora* and *A. musiformis* (Orbiliales) Liquid Culture Filtrates against Infective Larvae of the Sheep Blood-Feeding Nematode *Haemonchus contortus* (Trichostrongylidae)

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Abstract: The individual and combined effects of extracellular products (ECP) from *Arthrobotrys oligospora* (*Ao*) and *A. musiformis* (*Am*) growth in liquid media against *Haemonchus contortus* L3 (HcL3) were assessed. The following methodological steps were established: isolation, morphological (MI) and molecular identification (Mol-I), assessment of nematocidal activity (NA) of fungal liquid culture filtrates (LCF) produced in two liquid media alone and in combination and finally, the myco-compound profile identification (MCP). The MI of both isolates suggested that the fungi corresponded to the species *Ao* and *Am*. This result was confirmed by PCR analysis followed by sequencing, alignment and obtaining coverage and similarity with respect to isolates reported in the NCBI database. Likewise, the highest *Hc* mortalities were 91.4% with individual LCF of *Am* and 86.2% with those of *Ao* at the highest concentration (100 mg/mL) in Czapek-Dox Broth. The combination of both LCF resulted in a similarly high larval mortality with no statistical differences in relation to individual activity (P>0.05). The MCP showed the presence of alkaloids in both fungi. Coumarins, triterpenes and saponins were found only in *Ao*. Both fungi produced ECP with a high NA that could be identified and assessed in future studies as potential natural anthelmintic compounds.

Keywords: Nematophagous fungi; *Arthrobotrys*; *Haemonchus*; predation; myco-compounds; nematocidal activity

1. Introduction

Haemonchus contortus is a blood-feeding parasitic nematode living in the abomasum (stomach) of small ruminants that provokes gastritis, blood-loss in the abomasum, anorexia, anaemia, lethargy, weakness, weight loss, emaciation and, in severe cases, the death of young animals [1, 2]. This and other genera/species of parasitic nematodes live in the gastrointestinal tract of small ruminants, where they cause severe deterioration of flock health and productivity. Gastrointestinal nematodiasis is controlled by the frequent and continuous administration of chemical anthelmintic drugs to the animals; however, the development of anthelmintic resistance in the parasites occasions an increasing inefficacy of these drugs that has raised the alarm regarding the use of these drugs by farmers around the world [3, 4]. During recent decades, strategies other than the use of chemical anthelmintic drugs have been explored, including the use of natural antagonists of nematodes, such as a group of micro-fungi called nematode-trapping fungi [5, 6]. Nematode-trapping fungi are regular microorganisms

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from the soil mycobiota living as saprophyte organisms and taking their nitrogen and carbon sources from decaying wood and litter; however, they can transform their saprophyte lifestyle into a predatory or parasitic one in the presence of nematodes such that, once trapped, they are used as a food source by fungi [7]. The species *Arthrobotrys oligospora* and *A. musiformis* have been classified as Orbiliales fungi [8]. These species develop three-dimensional adhesive nets from their mycelia where nematodes are trapped and destroyed for eventual use by fungi as their main source of nutrients [9]. In addition to the mechanical capture exerted by trapping devices, nematode-trapping fungi possess other strategies to kill and penetrate nematodes using myco-chemical constituents, including enzymes and products derived from secondary metabolism [10]. The objectives of the present study were to isolate nematode-trapping fungi and perform a taxonomical identification via morphological and molecular procedures as well to assess the nematocidal activity of their liquid culture filtrates, either individually or combined, and produced in two liquid media (sweet potato dextrose broth [SPDB] and Czapek-Dox Broth [CzDB]) against *Haemonchus contortus* infective larvae and eventually to identify associated groups of myco-compounds.

2. Materials and Methods

2.1. Location

This study was performed at the Laboratory of Helminthology from the National Center of Disciplinary Research in Animal Health and Innocuity (CENID-SAI) in Jiutepec Municipality, Morelos State, Mexico. This centre belongs to INIFAP-Mexico (Agricultura, Mexican Government).

2.2. Nematodes

2.2.1. Obtaining the Free-Living Nematode Panagrellus redivivus for Use as Bait to Isolate Nematophagous Fungi

A population of the free-living nematode *P. redivivus* was provided as a fish food by a local pet store in Jiutepec Municipality, Morelos, Mexico. For *en masse* reproduction, nematodes were cultured in sterile plastic bowls containing sterile oat flakes (20 g), and 200 mL of sterile distilled water was added. Oat flakes and water were mixed and homogenized to finally obtain a humid mass that was used as a nutrient substrate for bacteria, and in turn, bacteria were used as the main source of food by the free-living nematodes. Bowls were covered with a cap of foil and gauze to prevent mosquitoes from entering. Cultures were incubated at room temperature (18–28 ˚C) for 7 days [11]. After the incubation period, some culture material was collected using a metal spoon and dissolved in a glass of water, producing many *P. redivivus* specimens swimming in an aqueous suspension. Nematodes were separated from the culture medium through a 74-µm sieve. This step was repeated several times until the nematodes were very clean. Nematodes were resuspended in sterile distilled water and sieved through a coffee filter. Nematodes were eventually recovered using the Baermann funnel technique [12].

2.2.2. Procurement of Haemonchus Contortus Infective Larvae (L3)

A lamb artificially infected with *H. contortus* was previously inoculated (*per os*) with 350 infecting larvae per kilogram body weight. After a pre-patent period of 21 days, faecal samples taken directly from the rectum of this lamb tested positive for the presence of nematode eggs by the McMaster technique. Faeces of the infected lamb were collected directly from rectum of this animal. All rules regarding the treatment of animals and the prevention of unnecessary animal suffering were carefully followed according the Norma Oficial Mexicana (Official Mexican Standard) with official rule number NOM-052-ZOO-1995 (http://www.senasica.gob.mx, accessed on 8 August 2023). Additionally, the Ley Federal de Sanidad Animal (Federal Law for Animal Health) DOF 07-06-2012 was strictly followed in accordance with the ethical standards outlined by INIFAP. Fresh faeces were ground in a plastic bowl and mixed with small pieces of polyurethane foam to obtain a porous mass that retained the oxygen necessary for the optimum development of nematode eggs [13] (Iliev et al.,

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2018). Faecal cultures were incubated at room temperature (18–25 °C) for 7 days. After 5–7 days of elaboration of faecal cultures a rather large amount of *H. contortus* infective larvae was collected using the Baermann Funnel technique for 24 h [13, 14]. Infective larvae of the parasite were cleaned by using the differential centrifugation technique with 40% sucrose density gradients [15]. After centrifugation for 5 min at 3500 rpm, a white ring in the interphase between water and sucrose corresponding to clean larvae was visualized. Larvae were removed with a Pasteur pipette and deposited into assay tubes, which were filled with water. In order to discard sucrose residue from the aqueous suspension of larvae, the assay tubes containing larvae in suspension were centrifuged at the same speed and spinning times, and larvae were sedimented to the base of the assay tubes. The supernatant was discarded and the tubes filled again with sterile water and centrifuged again. After three to four centrifugations, larvae in the sediment were free of sucrose residues.

2.3. Isolation of Nematophagous Fungi

Two 50-g samples of soil from a poultry farm in Cuernavaca Municipality, state of Morelos, Mexico were taken and transported in plastic bags to the Laboratory of Helminthology of CENID-SAI in Jiutepec, Morelos. A small soil sample (approximately 0.5 g) was sprinkled on sterile water agar plates. Plates were incubated at room temperature (18–25 °C) for three days. After this period, some drops of an aqueous suspension containing an undetermined number of specimens of the free-living nematode *P. redivivus* were added to each plate in order to promote the growth of nematophagous fungi. After one week, the agar surface was observed under the microscope, and aerial structures typical of nematophagous fungi were seen, including trapping devices, conidiophores and trapped nematodes. These structures were transferred to fresh sterile water agar plates using a sterile metal needle. Plates were maintained at the same temperature, and aerial structures were again transferred to sterile water agar plates. This process was repeated until fungi were eventually obtained in pure culture [16].

2.4. Procurement of Fungal Liquid Culture Filtrates

Fungi were cultivated in two different liquid culture media: SPDB and CzDB. Briefly, SPDB was prepared using 200 g of organic sweet potato (obtained from an organic farm at the Autonomous University of the State of Morelos (UAEM) in Cuernavaca, city). Sweet potato was peeled and chopped in small, square pieces of approximately 1 cm^2. Pieces of sweet potato were cooked in 1 L of distilled water and boiled for 25 min. Subsequently, cooked medium was sieved using a piece of gauze to separate the solid material, and 20 g of dextrose were added to the liquid phase and the volume adjusted to 1 L with distilled water. The liquid was transferred to 250-ml flasks, depositing 50 mL of media in each flask (n=3). Flasks containing the medium were sterilized in an autoclave and allowed to cool. Fifty hundred microliters of penicillin (50 I.U./mL)/streptomycin (50 µg/mL) were added to each flask. This procedure was performed using a laminar flue cabinet. Later on, three plugs (1 cm^2) obtained from the surface of a water agar plate containing a 21-day-old fungal culture were deposited in each flask. This procedure was performed for each of the two fungi. The same number of flasks with only liquid medium and without any fungal inoculum was used as a negative control. After the incubation period, liquid culture filtrates were obtained as follows: mycelia were separated by filtration using different kinds of filters and pore diameters, including a Whatman® #4 25 µm filter paper, and passed through three Millipore® filters (2 µm, 0.45 µm and 0.22 µm). Filtration was achieved using a Millipore filtration unit connected to a vacuum pump (Millipore High Performance Pump115 V/60 Hz, Darmstadt, Germany). Filtered media were concentrated using a rotatory evaporator (Buchi® R-300, Switzerland) to eliminate the greatest volume of water without altering the sample characteristics. Finally, evaporated material was lyophilised using a LABCONCO® FreeZone 4.5 plus, U.S.A lyophiliser.

2.5. Traditional Taxonomic Identification of Fungal Isolates by Morphometry

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The morphological identification of fungi was performed by both macroscopic and microscopic observations. In the case of macroscopic characteristics of both fungi growing in water-agar and potato dextrose agar were described. Regarding the microscopic characteristics of fungi, aerial structures such as conidia, conidiophores, trapping devices and the presence or absence of chlamydospores, among other characteristics, were observed and analysed under the microscope (20x and 40x). Twenty-five conidia and conidiophores were randomly taken, and their dimensions, i.e. height and width, were measured and recorded. Taxonomic morphometric identification was achieved by comparing the characteristics of our isolates with those described in specialized taxonomic keys [17, 18, 19]. Additionally, a set of microphotographs of the aerial structures of taxonomical importance were taken in a Leica DM6 B optical microscope.

2.6. Molecular Identification

Fungi were recovered from CzDB cultures and processed to obtain DNA using the Wizard® Genomic DNA Purification Kit (PROMEGA, USA). Genomic material was quantified using an IMPLEN (NanoPhotometer NP80) spectrophotometer. The endpoint PCR technique was performed following the procedures standardized at the Laboratory of Helminthology of CENID-SAI, INIFAP according to the methodology described by Tigano-Milani et al. (1995) [20]. The Internal Transcribed Spacer (ITS)-1, ITS-2 and 5.8S complete regions and a partial sequence of small 18S and large 28S sub-unit regions were amplified. The selected primers were ITS5-forward (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS4-reverse (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) [21].

The PCR technique is briefly described next. The reaction was carried out in a 20-µL total volume, containing 100 ng of gDNA, 10 µL GoTaq® Green Master Mix 2X (PROMEGA, USA), 1.5 µL of each primer at 20 µM and nuclease-free water to a total volume of 20 µL. The PCR method was carried out using a C1000 Touch® Thermal Cycler (BIORAD, Hercules, CA, USA). The PCR conditions were established as follows: initial denaturation at 94 °C for 3 min; an amplification stage, including 35 cycles of denaturation at 94 °C for 1 min, annealing at 42 ºC for 90 s, extension at 72 °C for 90 s; and a final extension stage at 72 °C for 5 min.

The Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) was used to purify the PCR products. Genomic material was sequenced at the Institute of Biotechnology of the National Autonomous University (IBT-UNAM), Cuernavaca city, Morelos, Mexico, using an Applied Biosystem Sequencer (7700; Thermo Fisher Scientific, Waltham, MA, USA). Similarity and coverage of sequences was achieved using the BLAST tool from NCBI. The sequences were compared with the closest sequences previously reported in the NCBI database. Molecular analysis was used to confirm the morphological identification [22].

2.7. Assessing the Predatory Activity of Fungi against Haemonchus Contortus Infective Larvae

A square piece of water agar (1 cm^2) from 10-day-old culture plates of *A. oligospora* was cut and deposited on the centre of a fresh sterile water agar plate (60 mm diameter). The same procedure was performed with *A. musiformis* (n=10). All plates were incubated at room temperature (18–25 °C) for 10 days. After this period, 10 other water agar plates without any fungus were prepared and used as a control group. Two hundred *H. contortus* infective larvae contained into 100 µL of PBS (7.2 pH) were individually added to each plate of the three experimental groups and incubated at the temperature mentioned above for 10 days. After incubation, the agar from every plate was individually transferred to a Baermann funnel apparatus where it remained for 24 h. The face of the agar on which nematodes and fungi were located was placed toward bottom of the assay tubes in order to allow non-trapped larvae to freely migrate and eventually remain sedimented at the bottom of the tubes. Non-trapped larvae from nematode/fungus interactions and whole larvae from control groups were recovered and quantified. The number of larvae in ten 5-µL aliquots from a 3-ml total volume recovered from experimental plates was quantified under a microscope (5×), and the mean numbers of larvae per group were estimated.

The percentage reduction attributed to the predatory effect exerted by fungi was estimated using the Abbott formula:

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$$Larval\ Reduction\ \% = \frac{(RLCgroup - RLinteraction)}{RLCgroup} 100$$

where:

$RLCgroup$ = *Recovered larvae from the control group*

$RLinteraction$ = *Recovered larvae from the fungi/larvae interaction group*

2.8. Assessing the Nematocidal Activity of Fungal Liquid Culture Filtrates against Haemonchus Contortus Infective Larvae

The interaction between Liquid Culture Filtrates (LCF) and *H. contortus* infective larvae was carried out using 96-well microtiter plates (n=4). Six experimental groups were established using three different concentrations of LCF in each treatment (100, 50 and 25 mg/mL) as follows: 1) *A. oligospora* LCF growth in SPDB; 2) *A. musiformis* LCF growth in SPDB; 3) The combination of LCF from both fungi (using half of the volume used in individual LCF). Likewise, groups 4, 5 and 6 were similar to 1, 2 and 3, but LCF of fungi growing in CzDB were used instead of SPDB. Additionally, two groups containing only the SPDB and CzDB media were used to discard any possible lethal effect of these media on the nematodes. Likewise, a group with only PBS and one with 0.5% commercial ivermectin (Ivomec®) were used as negative and positive controls. LCF previously dried were dissolved in PBS (pH=7.2). Fifty microliters of the corresponding filtrate and 50 µL of an aqueous larval suspension (in PBS) containing approximately 100 *H. contortus* infective larvae were deposited in every one of the four wells per treatment. All plates were incubated at room temperature (18–28˚C) for 72 h (Figure 1).

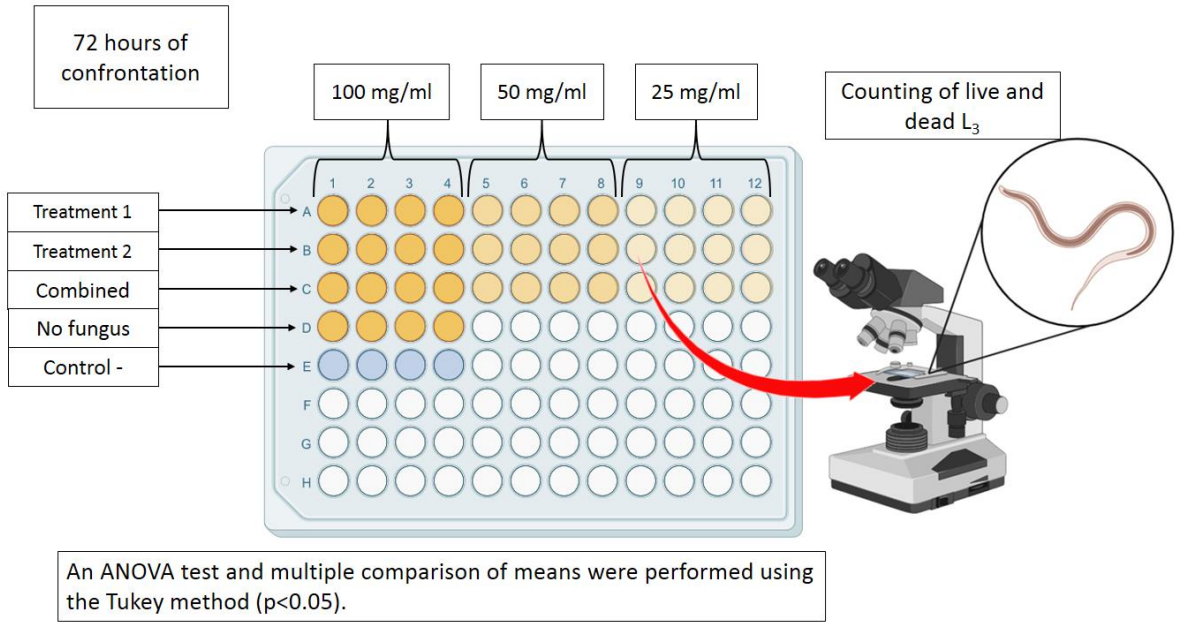

Figure 1. Scheme representing the steps of the experimental process to evaluate the lethal effect of liquid culture filtrates obtained with two nematophagous fungi *Arthrobotrys oligospora* and *A. musiformis* growth in two culture media, Sweet Potato Dextrose Broth and Czapek-Dox Broth, against *Haemonchus contortus* infective larvae.

After incubation, the volume of each well was observed under the microscope, and dead and live larvae were counted following the previously described procedure. Motionless larvae were determined to be alive or dead by applying a physical stimulus by touching its cuticle using a sharp metallic needle. Live larvae normally start to move actively after this stimulus. Larvae that remain motionless after this stimulus are considered as dead larvae [23]. The whole experiment was repeated three times. Larval mortality obtained for each treatment and control group was calculated. The larval

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mortality attributed to the effect of LCF of each fungus and each concentration was estimated using the following formula:

$$\%\ Larval\ Mortality = \frac{(Mortality\ L3\ Treated\ group - \ Mortality\ L3\ Control\ group)}{(1 - Mortality\ L3\ Control\ group)} 100$$

where:

$Mean\ L3\ Treated\ group$ = *Mean of larval mortality from treated group.*

$Mean\ L3\ Control\ group$= Mean of larval mortality from control group.

2.9. Microscopic Analysis of Haemonchus Contortus Infective Larvae Exposed to Fungal Liquid Culture Filtrates

After exposure of *H. contortus* larvae to LCF, a random selection of larvae from the different treatments was carried out in order to photograph possible morphological changes attributed to the effect of compounds present in the fungal filtrates. Larvae were photographed using a LEICA DM6 compound microscope using the program LAS V4.9 to document our findings.

2.10. Myco-Chemical Profile

A myco-quantitative reagent analysis was carried out using standard procedures with the proper reagents and methods. Alkaloids were determined using Dragendorff Mayer and Wagner´s reagents, and the Bornträger test was performed to identify the presence of coumarins. Likewise, the presence of flavonoids was investigated using Mg^{2+} and HCl tests. On the other hand, the ferric chloride, gelatine and saline solution tests were carried out to identify tannins. Finally, the Lieberman–Burchard and Salkowski tests were used to identify the presence of Triterpenes [24].

2.11. Statistical Analysis

Data obtained from the predatory activity assay of both fungal isolates were individually analysed by the Student's *t*-test, where the larval reduction percentage was obtained by comparing the mean number of recovered larvae from the nematode/fungi interaction plates and from the control group without fungus. For the results of the nematocidal activity of LCF, a completely random model was used, and ANOVA was performed where the means of larval mortality in the different treatments was considered as the dependent variable. An orthogonal contrast by the Bonferroni method was performed to compare the effect of the combination of both LCF from fungi grown in the same media using the following coefficients: 0.5(AspT) + 0.5(AspG) – 1(combined, 50:50). A p value of 0.05 was used for all tests.

3. Results

3.1. Traditional Taxonomy (Morphometrics)

After the main structures of taxonomic interest were observed and analysed under the microscope (including conidia and conidiophore measurements) the morphological characteristics were compared with those described in taxonomic keys, and the authors eventually decided to classify these fungi as *A. oligospora* and *A. musiformis*. The isolate AspT showed hyphae, conidia and hyaline conidiophores with repeated proliferation of conidia. Conidia showed a globose shape, lightly constricted in their septum. This fungus showed the presence of three-dimensional adhesive nets (Figure 2).

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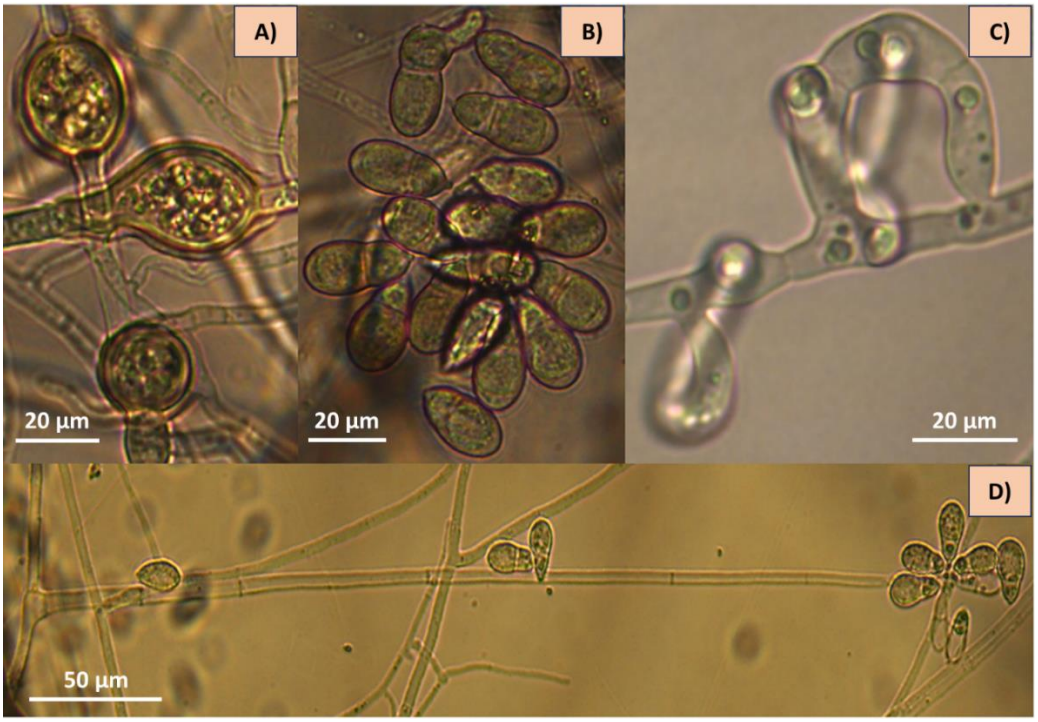

Figure 2. Microphotographs of *Arthrobotrys oligospora* showing structures of taxonomic importance: A) chlamydospores, B) conidia, C) three-dimensional adhesive nets and D) conidiophore.km.

Likewise, the analysis of the isolate AspG showed hyaline hyphae, conidia and conidiophores. Nevertheless, conidiophores showed long and cylindrical denticles, produced by candelabra, where conidia were generated. Conidia were elongated and slightly curved, typical of *A. musiformis*. This isolate showed the same trapping devices corresponding to the three-dimensional adhesive nets described above (Figure 3).

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Figure 3. *Arthrobotrys musiformis* structures of taxonomic importance: A) Candelabrum; B) Three-Dimensional adhesive net; C) Conidia; D) Conidiophore and E) Chlamydospores.

The summarized information about measurements and observations of the main morphological characteristics of both isolates are shown in Table 1.

Table 1. Main morphological characteristics observed in the two isolates of nematophagous fungi belonging to the genus *Arthrobotrys* and means of measurement of taxonomically important structures.

Characteristic	Strain 1 (AspT)	Strain 2 (AspG)
Conidium shape	Globose, slightly constricted in the septum (in occasions).	Ellipsoidal to obovoidal, slightly curved
Septum	One septum situated slightly below the half conidium	One septum situated slightly below the half conidium
Number of conidia per conidiophore	6 (4-8)	7 (3-11)
Conidia length (µm)	21.1 (19-23)	32.7 (27-40)
Conidia width (µm)	11.4 (10-12)	13 (11-17)
Conidiophores length (µm)	344 (193-429)	291 (141-403)
Identified species	*Arthrobotrys oligospora*	*Arthrobotrys musiformis*

3.2. Molecular Identification

Once nucleotide sequences from both fungi were analysed by alignment with the sequences previously reported in the NCBI database, query covers and similarity percentages were obtained (Tables 2 and 3). The analysis shows a high similarity (98.52%–99.54%) between the strain AspT and

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sequences from the species *A. oligospora*. On the other hand, sequences from AspG had high similarity with the species *A. eryuanensis* (MT612105.1) and *A. musiformis* (99.32% for both species).

Table 2. Query covers and similarity percentages of the isolate (AspT) (*Arthrobotrys oligospora*) in relation to the first five isolates found in the NCBI-Blast alignment.

Isolate (Genus/Species)	Query cover %	Similarity %	Gen Bank Accession number NCBI
Orbilia oligospora	99	99.41	OQ781152.1
O. oligospora	98	98.86	MZ427471.1
Arthrobotrys oligospora	96	99.54	MF948413.1
O. oligospora	97	98.95	ON114061.1
A. oligospora	99	98.52	KC663625.1

Table 3. Query covers and similarity percentages of the isolate (AspG) (*Arthrobotrys musiformis*) in relation to the first five isolates found in the NCBI-Blast alignment.

Isolate (Genus/Species)	Query cover %	Similarity %	Gen Bank accession number
Arthrobotrys sp.	96	99.49	ON383425.1
A. eryuanensis	96	99.32	MT612105.1
A musiformis	96	99.32	MH855842.1
A musiformis	96	99.32	KP859624.1
A. musiformis	96	99.32	OL454931.1

3.3. Predatory Activity Assessment of the Two Nematophagous Fungal Isolates against Haemonchus Contortus Infective Larvae

The mean numbers of *H. contortus* infective larvae recovered from agar plate groups with and without fungi are shown in Table 4.

Table 4. Results of the predatory activity of two nematophagous fungi *Arthrobotrys oligospora* (AspT) and *A. musiformis* (AspG) against *Haemonchus contortus* infective larvae on water agar plates.

Isolate (species)	Recovered larvae Control group (Mean ± SE)	Recovered larvae Treated group (Mean ± SE)	Larval reduction %
Arthrobotrys oligospora	192 ± 38.21	105 ± 26.42	45.14 [a]
Arthrobotrys musiformis	197 ± 60.53	57 ± 30.35	70.95 [b]

[a,b] Different letters show statistical differences between groups.

3.4. Microscopic Findings

Haemonchus contortus infective larvae trapped in three-dimensional adhesive nets of *A. oligospora* and *A. musiformis* are shown in Figures 4A and 4B, respectively. In both images, hyphae are observed invading the nematode corps.

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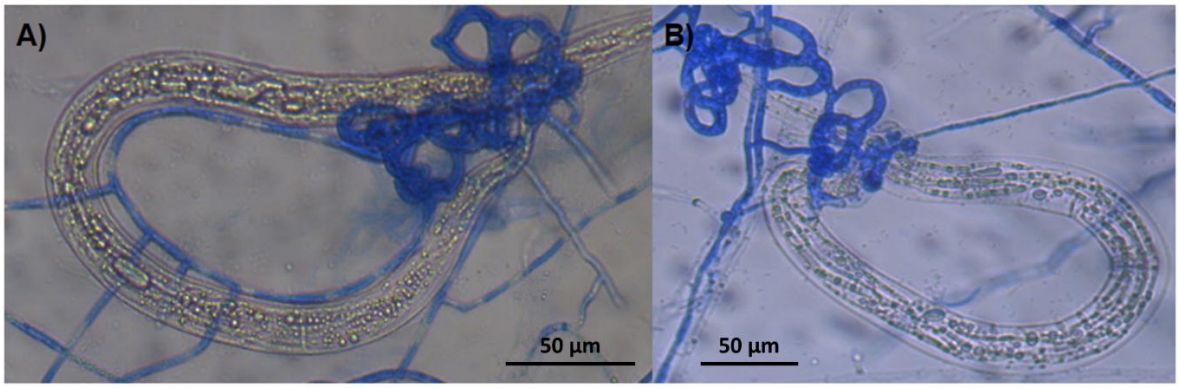

Figure 4. Aspect of *Haemonchus contortus* infective larvae captured in three-dimensional adhesive nets of two nematophagous fungi, *Arthrobotrys oligospora* (A) and *Arthrobotrys musiformis* (B).

3.5. Assessment of the In Vitro Nematocidal Activity of Liquid Culture Filtrates of Arthrobotrys oligospora and Arthrobotrys musiformis Produced in SPDB and CzDoxB against Haemonchus contortus Infective Larvae

The results regarding the larval mortality of *H. contortus* attributed to the effect of LCF of both nematophagous fungi either individually or in combination (50:50) grown in the two evaluated media are shown in Tables 5 and 6, respectively.

Table 5. Mean of *Haemonchus contortus* dead and total recovered larvae and mean larval mortality percentages after 72 h exposure to *Arthrobotrys musiformis* and *A. oligospora* liquid culture filtrates obtained from Sweet Potato Dextrose Broth and the combination of both fungi.

Concentration (mg/mL)	Fungal filtrate	DL	TL	Mortality %	SE	Significance *
0	*A. musiformis*	6	119	5.03	0.87	1
	A. oligospora	6	119	5.03	0.87	
	Combination	6	119	5.03	0.87	
25	*A. musiformis*	15	107	13.70	3.24	0.072
	A. oligospora	12	96	12.14	2.74	
	Combination	21	101	20.54	3.95	
50	*A. musiformis*	25	104	24.50	5.08	0.456
	A. oligospora	31	104	29.83	6.03	
	Combination	19	86	22.18	5.06	
100	*A. musiformis*	83	110	75.55	5.37	0.543
	A. oligospora	82	104	78.29	2.65	
	Combination	79	99	79.84	3.04	

* Significance of combined effect of filtrates testing the contrast C = 0.5 + 0.5 − 1. DL= Dead larvae; TL=Total larvae; SE=Standard Error.

Table 6. Mean of *Haemonchus contortus* dead (DL) and total recovered larvae (TL) and mean larval mortality percentages after 72 h exposure to *Arthrobotrys musiformis* and *A. oligospora* liquid culture filtrates obtained from Czapek Dox Broth and the combination of both fungi.

Concentration (mg/mL)	Fungal filtrate	DL	TL	Mortality %	SE	Significance *
0	A. musiformis	2	86	2.65	0.40	0.588
	A. oligospora	2	80	2.12	0.38	
	Combination	2	80	2.12	0.38	
25	A. musiformis	22	81	27.08	3.42	0.011

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	A. oligospora	23	86	27.34	5.75	
	Combination	37	84	44.07	5.75	
50	A. musiformis	44	86	51.59	5.54	0.916
	A. oligospora	38	89	43.03	8.84	
	Combination	36	75	48.29	7.96	
100	A. musiformis	83	91	91.35	3.05	0.308
	A. oligospora	75	87	86.17	3.39	
	Combination	81	87	92.55	2.47	

* Significance of synergistic effect of filtrates C = 0.5 + 0.5 – 1. DL= Dead larvae; TL=Total larvae; SE=Standard Error.

On the other hand, the comparison between the larval mortality obtained in the different LCF concentrations with both fungi and both media (SPDB and CzDB) are shown in Tables 7 and 8, respectively.

Table 7. Results of *Haemonchus contortus* larval mortality percentages produced by *Arthrobotrys musiformis* and *A. oligospora* liquid culture filtrates obtained in Sweet Potato Dextrose Broth at different concentrations, either individually or combined.

Fungus	Concentration (mg/mL)	Mean ± SE	Significance *
A. musiformis	0	5.03 ± 0.87	a
	25	13.70 ± 3.24	ab
	50	24.50 ± 5.08	b
	100	75.55 ± 5.37	c
A. oligospora	0	5.03 ± 0.87	a
	25	12.14 ± 2.74	a
	50	29.83 ± 6.03	b
	100	78.29 ± 2.65	c
Combination	0	5.03 ± 0.87	a
	25	20.54 ± 3.95	b
	50	22.18 ± 5.06	b
	100	79.84 ± 3.04	c

Same letters indicate no statistical differences among the different concentrations. Tukey ($p<0.05$).

Table 8. Results of *Haemonchus contortus* larval mortality percentages produced by *Arthrobotrys musiformis* and *A. oligospora* liquid culture filtrates obtained in Czapek-Dox Broth at different concentrations either individually or combined.

Fungus	Concentration (mg/mL)	Mean ± SE	Significance *
A. musiformis	0	2.65 ± 0.40	a
	25	27.08 ± 3.42	b
	50	51.59 ± 5.54	c
	100	91.35 ± 3.05	d
A. oligospora	0	2.12 ± 0.38	a
	25	27.34 ± 5.75	b
	50	43.03 ± 8.84	b
	100	86.17 ± 3.39	c
Combination	0	2.12 ± 0.38	a
	25	44.07 ± 5.75	b
	50	48.29 ± 7.96	b
	100	92.55 ± 2.47	c

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Same letters indicate no statistical differences among the different concentrations. Tukey ($p<0.05$).

3.6. Microscopic Findings Regarding Haemonchus contortus Infective Larvae Exposed to Liquid Culture Filtrates of Two Nematophagous Fungi Grown in Sweet Potato Dextrose Broth and Czapek-Dox Broth

A set of microphotographs showing morphological changes identified in *H. contortus* infective larvae after exposure to liquid culture filtrates of *A. musiformis* and *A. oligospora* are shown in Figure 5. Images A and C show the aspects of *H. contortus* infective larvae after 72 h exposure to LCF of *A. musiformis* and *A. oligospora*, respectively (grown in CzDB) assessed at 100 mg/mL. In these images a thickening of the larval bodies was observed in some areas; meanwhile, in other areas a diminishing of the body thickness was visualized. In both cases a clear loss of the intestinal cellular structural architecture was observed (c).

On the other hand, larvae exposed to LCF of both fungi grown in SPDB (B) and (D) showed similar damage, but more severe in SPDB, and a general deformation of larval bodies with a slimming in some areas of the nematode corps (a) and lose of turgor body in other areas (b) was observed.

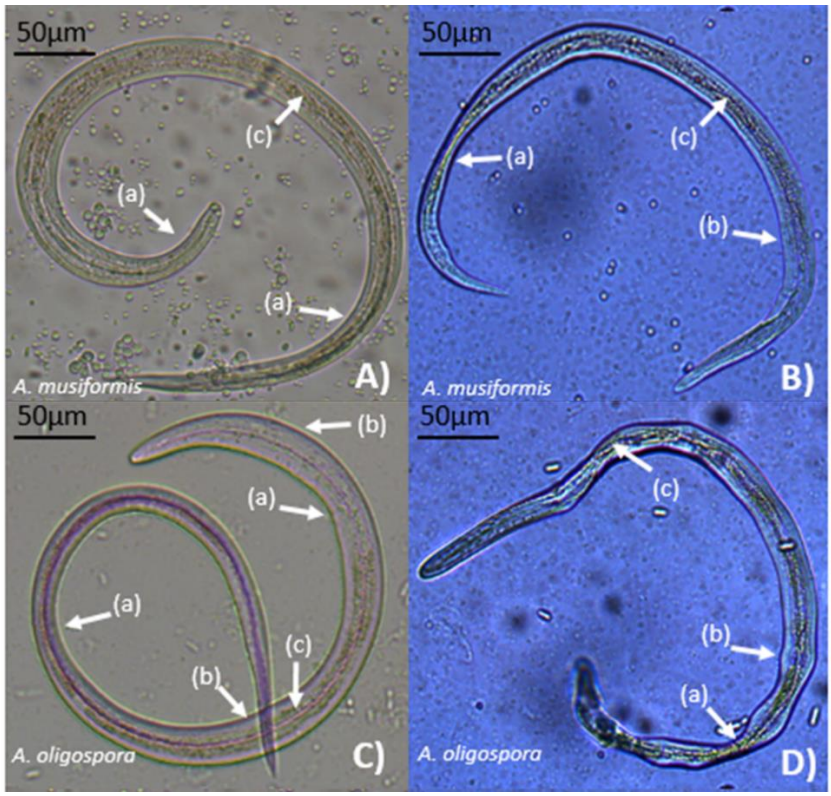

Figure 5. Microphotographs showing the aspect of *Haemonchus contortus* infective larvae after 72 h exposure to liquid culture filtrates obtained with two nematophagous fungi *Arthrobotrys musiformis* and *A. oligospora* grown in Czapek-Dox Broth (A) and (C) and Sweet Potato Dextrose Broth (B) and (D).

3.7. Myco-Chemical Compound Profile

The results of the myco-quantitative reagent analysis obtained with LCF of both fungi in both culture media are shown in Table 9.

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Table 9. Mycochemical profile obtained from liquid culture filtrates of two nematophagous fungi *Arthrobotrys musiformis* and *A. oligospora* grown in two culture media, Czapek-Dox Broth and Sweet Potato Dextrose Broth, by myco-quantitative reagent analysis.

Metabolites and reagents	Colorimetric Reaction	*Arthrobotrys musiformis*		*Arthrobotrys oligospora*	
		CzDoxB	SPDB	CCzDox	SPDB
Alkaloids: Dragendorff	Turbidity or precipitate (Red, to orange, white to cream or brown)	+	+	+	+
Mayer		+	+	+	+
Wagner		+	+	+	+
Coumarins: Bornträger	Yellow florescence (UV)	-	+	+	+
Flavonoids: Mg^{2+} and HCL	Red, orange or violet	-	-	-	-
Tannins	Hydrolizables (blue)	-	-	-	-
Iron chloride ($FeCl_3$)	Condensaded (green)	-	-	-	-
Confirmation					
Gelatine solution	White precipitate	-	-	-	-
Gelatine and saline solution	White precipitate	-	-	-	-
Saline solution	White precipitate	-	-	-	-
Triterpenes/Sterols: Liebermann-Buchard	Blue, green-blue (Sterols)	-	-	-	-
Salkowski	Red to purple (triterpene)	-	+	+	+
Saponins: Water	Foam formation	-	+	+++	++

-: undetected reaction; **+**: slightly positive reaction; **++**: positive reaction; **+++**; strong positive reaction.

4. Discussion

4.1. Traditional Taxonomy through Morphological Characteristics

At first sight, the growth of erect and long-stem apical conidiophores crowned by conidia clusters of globose form, as well as the presence of one septum almost at the middle of the conidia in the Asp-T strain suggested to us the presence of a nematophagous fungus belonging to the genus *Arthrobotrys* [18]. However, there are several species sharing these characteristics, and this fact can cause confusion in the taxonomic identification. Some of the species sharing these similar characteristics are *A. oligospora*, *A. robusta*, *A. superba*, *A. conoides* and *A. arthrobotryoides*, among others. After observing the characteristics in more detail, including the measurements of some structures, i.e. conidia length and width, the number of conidia in the clusters, the presence or absence of branched conidiophores, the presence or absence of chlamydospores and particularly, the type of trapping devices, we were able to differentiate our isolate from other species. Because our isolate showed only unbranched conidiophores, and the measurements of conidia and the length of conidiophores were similar to those of species described in the taxonomical identification keys, we were able to discard several species that do not share these characteristics.

Additionally, our isolate showed an important taxonomical characteristic that is the type of conidiophore at the top, precisely where the conidiophore produces denticles where conidia are generated (conidiogenesis). Such denticles are typical of Arthrobotryoid conidiophores (Figure 6A), and this characteristic differs from those of other species such as *A. musiformis* and *A. javanica* that, instead of these denticles, form a candelabrum where conidia are produced. The presence of conidiophores with denticle formation is typical of the species *A. oligospora* (Figure 6). It is important to mention that this isolate showed the presence of chlamydospores; as well as the formation of three-

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dimensional adhesive nets as trapping devices. These characteristics led us to classify our isolate as *A. oligospora* on the basis of morphological analysis. Regarding the other fungal isolate recorded as Asp-G, this isolate showed a conidia cluster different to that of *A. oligospora*. Instead, this fungus showed the presence of 4–8 obovoidal, elongated, slightly curved and slightly constricted conidia. There was a septum slightly situated below the middle of conidia. Elongated conidia were arranged laterally or slightly above the conidiophores. This isolate also showed the presence of the same trapping devices as *A. oligospora*, three-dimensional adhesive nets. This fungus also showed the presence of chlamydospores but possessed candelabrelloid conidiophores typical of *A. musiformis*. This structure resembles an elk horn where conidia are developed (Figure 6B).

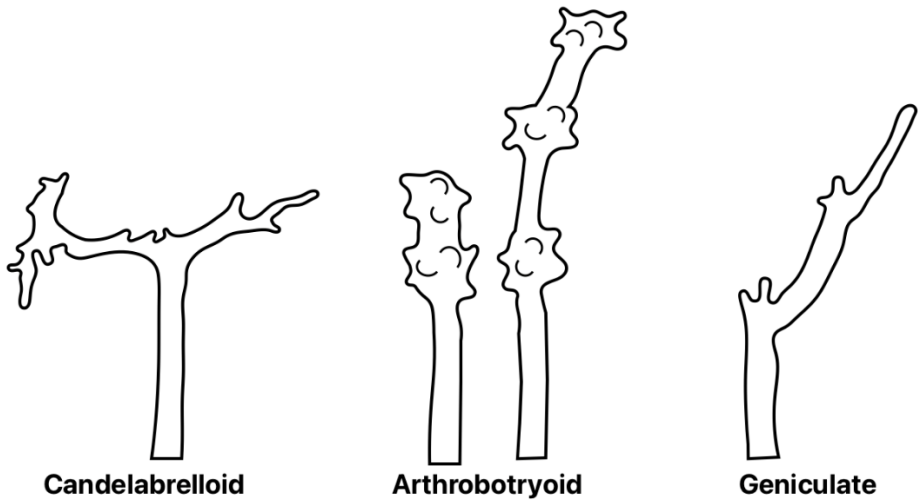

Figure 6. Aspects of different types of apical extremes of conidiophores of different nematophagous fungi.

4.2. Molecular Taxonomy

After analysing the DNA sequences of the AspT strain and comparing this information with the sequences of isolates previously reported in the NCBI database, high coverage (96%–99%) and similarity (98.86%–99.54%) values with respect to fungi from the species *Orbilia oligospora* and *A. oligospora* were found. It seems that both species are synonyms according to recent nomenclature reported in the NCBI database [25] (Taxonomy ID: 2813651, NCBI:txis2813651). On the other hand, *A. musiformis* matched those sequences previously reported in the NCBI database with high levels of coverage (99.32) corresponding to *A. musiformis* (MH855842.1., KP859624.1, OL454931.1) and *A. eryuanensis* (MT612105.1). However, there are some remarkable morphological differences between *A. musiformis* and *A. eryuanensis*. For example, the length of erect conidiophores in *A. musiformis* can reach up to 900 µM [18]; meanwhile, *A. eryuanensis* reach a length range of 110–308 µm, and this species also produces macro- and microconidia [19].

4.3. Nematode-Predatory Activity of Fungal Isolates

The results regarding the predatory activity assessment show reduction percentages of *H. contortus* infective larvae attributed to the predatory effect of both species of nematophagous fungi: moderate (around 45%) in *A. oligospora* and higher than 70% in *A. musiformis*. In the literature consulted, we found several reports where different strains of *A. musiformis* obtained from different sources showed variable results in terms of their predatory activity against ruminant parasitic nematodes, i.e. Cao et al. (2018) [26], using Chinese isolates of this species, reported a range of 89.02% to 94.80% reduction of infective larvae of the nematode *Trichostrongylus colubriformis* [26]. Likewise, an *A. musiformis* strain isolated from a soil sample from Cuautla municipality, Morelos state, Mexico

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showed 71.54% larval mortality using *H. contortus* (L3) as a target [22]. Other isolates of *A. oligospora* and *A. musiformis* have shown variable results regarding predatory capability against infective larvae of ruminant parasitic nematodes and other nematode targets (Table 10). In this regard, we can build a reflection, the biological behaviour of nematophagous fungi is a dynamic process and it depends on the adaptation to environmental circumstances of each microorganism from the soil microbiota; such variation in the ability to form traps and capture nematodes of different taxonomic groups can be attributed to the influence of diverse abiotic and biotic factors in fungal habitats [27].

Table 10. Results of the in vitro predatory activity of *Arthrobotrys musiformis* and *A. oligospora* obtained from different sources and using different nematode targets.

Isolate	Source of Isolation	Nematode target	Predatory activity %	Author (s)
A. oligospora	Not available	*Meloidogyne incognita*(**)	79.6–87.5	[27]
A. musiformis	Mossy soil, decaying plant material (a rotten trunk) and soil containing Brahea palm roots	*H. contortus* (L3)	>97	[28]
A. oligospora	Faeces of water buffalo	*H. contortus* (L3)	>89	[29]
A. oligospora	Soil and animal faeces	*Panagrellus redivivus* (*)	57.2	[16]
A. musiformis	Soil sample	*H. contortus* (L3)	>74	[30]
A. oligospora	Soil samples	*Aphelenchoides besseyi*, *Bursaphelenchus xylophilus* and *Ditylenchus destructor* (**)	54.6–97.3	[31]

(*)=A Free-living nematode; (**)= Plant parasitic nematodes.

4.4. Nematocidal Activity of Fungal Liquid Culture Filtrates

When liquid culture filtrates of both fungi were used individually at the highest concentration (100 mg/mL) in the two assessed media, significant larval mortality was obtained. It is interesting that in both cases, when LCF were used, either individually or in combination, similar mortality values were found, ranging from 75.6% to 92.6%. No difference in the effect of individual or combined LCF was observed, since no statistical difference was observed in any of the cases. Although the concentration of LCF at which a lethal effect against *H. contortus* was observed can be considered high, is clear that one or more mycocompounds derived from the secondary metabolism of both fungi are responsible for the nematocidal activity. This result is important, since it is the first step of compound purification using chromatographic procedures, i.e. gas chromatography–mass spectrometry (GCeMS), following a bio-guided assay to identify potential bioactive molecules as natural de-wormers against ruminant parasitic nematodes [30, 32]. The technique of nuclear magnetic resonance could be an alternative method to identify the bioactive compound, although this procedure requires highly purified fractions [33]. After a rigorous study on the safety of administration in animals, followed by its innocuity to human beings and the absence of a negative environmental impact, these compounds could have significant implications as natural anthelmintics to at least partially replace the use of chemically synthesized, commercially available drugs. Regarding the synergistic effect that we originally proposed as a hypothesis, we did not find this effect, since LCF of both fungi showed an important lethal effect against nematodes at the highest assessed concentration.

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It is important to mention that at concentrations lower than 100 mg/mL, the results showed wide variability with very low ranges of lethality, in comparison with the highest concentration. The LCF of *A. oligospora* at 25 mg/mL in SPDB showed only 12.1% lethality; meanwhile, the highest lethality of *A. musiformis* (>51%) was observed at 50 mg/mL in CzDoxB. The large variability in the results at 25 and 50 mg/mL did not allow us to build a solid criterion about the bioactivity of these LCF.

4.5. Microscopic Analysis of Haemonchus contortus after Exposure to Fungal Liquid Culture Filtrates

After analysing the images captured by microscopy, we observed malformations in different sites of the larval bodies exposed to both LCFs. The larvae exposed to LCF from both fungi obtained from CzDoxB showed some morphological changes; however, the damage to those larvae exposed to LCF obtained from both fungi in SPBD showed more severe morphological alterations. These findings suggest that the culture media can influence the production of bioactive mycocompounds derived from the secondary metabolism of fungi. In future studies, we plan to use confocal laser scanning microscopy to perform a co-localization analysis to determine the site of action and the damage caused by metabolites responsible of the nematocidal activity [34].

4.6. Mycochemical Compound Profile

The groups of secondary metabolites found after mycochemical compound analysis of LCF obtained in both liquid media (CzDoxB and SPDB) of the two nematophagous fungi (*A. musiformis* and *A. oligospora*) were alkaloids with a light reaction in the four treatments: coumarins with a light reaction in *A. musiformis* in SPDB and in *A. oligospora* in both media. Regarding the presence of triterpenes, this group of compounds was found with a light reaction in LCF of *A. musiformis* in SPDB and in *A. oligospora* in CzDoxB; however, in *A. oligospora* a positive reaction was found in SPDB and a strong reaction in CzDoxB. These findings show that *A. oligospora*, despite not showing high predatory activity of *H. contortus* infective larvae using physical mechanisms (three-dimensional adhesive nets), in certain respects, compensate for this lack of a strong predatory activity by producing a large number of secondary metabolites with nematocidal activity, which could contribute to immobilization and killing of nematodes.

5. Conclusions

In the present study, no difference was found in the nematocidal activity of LCF of *A. oligospora* and *A. musiformis*, used individually or in combination, against *H. contortus* infective larvae. Liquid culture filtrates of both fungi exerted an important nematocidal effect at the highest concentration (100 mg/mL) when used either individually or in a combined manner. The results of the present study contribute important information about the predatory activity of these species of nematophagous fungi and additionally about the nematocidal activity of LCF produced by these fungi that could have implications for future studies exploring the use of this LCF or the metabolites responsible for nematocidal activity as potential tools of control against sheep haemonchosis.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, PMG and GPA; methodology, ACP; software, GABG; validation, AOJ and EGM.; formal analysis, EJDN; investigation, ACP; resources, PMG; data curation, AOJ and GPA; writing—original draft preparation, PMG; writing—review and editing, PMG and GPA; supervision, PMG and GPA; project administration, PMG; funding acquisition, PMG. "All authors have read and agreed to the published version of the manuscript."

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Institutional Review Board Statement: The animal that acted as an egg donor of the parasite was maintained under indoor conditions, following the rules of care, respect and animal welfare and especial attention was put in avoiding any unnecessary animal suffering. These conditions were strictly performed according to the Good Management Practices policies established at INIFAP. The Norma Oficial Mexicana (Official Mexican Standard) with official rule number NOM-052-ZOO-1995 (http://www.senasica.gob.mx, accessed on 6 February 2024) and the Ley Federal de Sanidad Animal (Federal Law for Animal Health) DOF 07-06-2012 were strictly applied

(https://www.gob.mx/cms/uploads/attachment/file/1187 61/LFSA.pdf, accessed on 6 February 2024) in accordance with the ethical standards outlined by INIFAP.

Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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