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In vivo anthelmintic effects of polyphenol-rich extracts from the salt-tolerant species *Cladium mariscus* (L.) Pohl, 1809 and *Limoniastrum monopetalum* (L.) Boiss., 1848 against gastrointestinal parasitic nematodes in lambs

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Abstract

Background Parasitic gastrointestinal infections caused by strongyle nematodes in grazing small ruminants endure as main cause of reduced animal productivity, health and welfare, compromising farm economy. For several decades, synthetic anthelmintics have been the cornerstone to control such parasites and limit their consequences but increasing issues regarding parasite resistances and concerns of chemical residues in the food chain, led to the exploration of novel approaches within the concept of integrated management solutions. Among them, bioactive plants and their associated secondary metabolites, have been increasingly explored. Recently, promising in vitro results were obtained with acetone aqueous extracts of polyphenol-rich salt-tolerant plants, namely *Cladium mariscus* (L.) Pohl, 1809 and *Limoniastrum monopetalum* (L.) Boiss., 1848. The current study aims to obtain the first proof of concept of these two plant extracts' in vivo anthelmintic value, relying on prior in vitro data.

Methods To assess the effects of drenching the plant extracts in *Haemonchus contortus* and *Trichostrongylus colubriformis* mixed infections, twenty lambs were divided into three experimental groups: a control group (C), a *C. mariscus* treated group (CM) and a *L. monopetalum* treated group (LM). Lambs were infected (DPI 0), drenched with extracts (DPI 30) and the experiment ended on DPI 48. Blood and fecal samples were collected regularly to assess parasitological and pathophysiological measurements.

Results Overall, results do not indicate significant in vivo parasitological effects for the two plant extracts, in the studied conditions. However, significant differences in PCV values were observed between the treated and control groups, hypothesizing potential effects on the overall animal health status, to be explored in future work.

Conclusions This work outcomes are discussed based on the available in vivo anthelmintic works using plant extracts, and methodological challenges are appraised.

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1 Background

Parasitic gastrointestinal nematodes (GIN) infections are widely present small ruminant outdoor systems, reliant on pastures or browses, representing a risk of infection due to the ingestion of infective third-stage larvae (L3). These GIN infections pose a significant economic and health burden, impacting animal health and productivity, especially those infected with the hematophagous parasite species *Haemonchus contortus*, which can present severe clinical symptoms, often leading to high mortality rates [1, 2].

GIN control has relied mostly on the persistent administration of synthetic anthelmintic drugs; however, the sole dependence on this chemical approach is nowadays facing strong restrictions. Firstly, due to growing concern over drug residues in animal-derived food products (e.g., meat and milk) and possible consequences to consumers' health [3]. Secondly, the long-term and intensive use of anthelmintic drugs culminated in the development of resistance to different commercial drug families [3, 4], a phenomenon that is expanding worldwide, including the rise of multi-resistant isolates, as illustrated by Rose Vineer and colleagues [4].

To address these issues, the exploration of different solutions has gained momentum in the last decades, within the novel concept of integrated management and control of GIN in ruminants, in a more sustainable manner—the so-called “basket of options” [3, 5, 6]. This approach focuses on three critical stages for the parasite's success: (1) the biology and dynamics of worm populations; (2) enhancement of the host's resistance and resilience to GIN, and (3) reducing pasture infectivity and limiting contact between the host and infective L3 larvae [3, 5, 6].

Within this framework, bioactive plants and secondary metabolites have gained increasing attention: plant diversity, influenced by their geographic and climatic distribution, along with their phylogenetic and chemical variety, presents an opportunity for novel compound discovery in nature. Plants represent a natural reservoir of compounds pending scientific validation and utilization, but promising to offer valuable nutraceuticals, phytotherapeutic agents, and novel scaffolds for chemical synthesis. Hoste and colleagues (2008) have put forward a methodical framework for identifying and selecting bioactive plants and derived compounds to contribute to the GIN control in ruminants

[7]. Within this conceptual frame, the model of bioactive plants, particularly Fabaceae family members containing polyphenols, has led to a bulk of studies on their in vitro and in vivo anthelmintic properties [8–11].

Recently, polyphenol-rich salt-tolerant plants were also identified, based on prior existing knowledge from ethnoveterinary practices and phytochemical studies [12, 13]. In vitro studies confirmed the inhibitory effects of acetone aqueous extracts of four salt-tolerant species, *Pistacia lentiscus* L., 1753, *Helychrisum italicum* subsp. *picardii* (Boiss. & Reut.) Franco, 1984, *Cladium mariscus* (L.) Pohl, 1809, and *Limoniastrum monopetalum* (L.) Boiss., 1848, on L3 larvae exsheathment and egg hatching processes of two GIN species, linked to the occurrence of different bioactive phenolic metabolites, possibly involved in the anthelmintic effect [13]. Within these four plants, additional investigations have been dedicated to *Cladium mariscus* (L.) Pohl focused on the variations in the anthelmintic effects and phytochemical contents, considering phenological and environmental factors [14, 15].

Translating in vitro results to in vivo settings remains a major challenge in the discovery pipeline of novel bioactive resources against GIN. Despite this difficulty, in vivo trials remain essential for validating the anthelmintic efficacy of botanical products and anthelmintic drugs. Unlike monogastric animals, ruminants present a more complex gastrointestinal system, increasing the difficulties of replicating those conditions under in vivo conditions. This makes testing on ruminant animals the ultimate proof of concept [16].

Therefore, based on previous in vitro data, the current study was designed to gather first-time in vivo data on the anthelmintic properties of *C. mariscus* and *L. monopetalum* acetone aqueous extracts. In line with this, efforts were directed toward the following three specific objectives: (1) to characterize the phytochemical composition of the two salt-tolerant plant extracts; (2) to analyze the effects of the oral administration of the extracts on GIN populations (egg excretion and egg development to infective larvae) and (3) its impact on lamb resilience by appraising the pathophysiological outcomes on the host.

2 Methods

2.1 Preparation and characterization of the plant extracts

2.1.1 Plant collection and processing

Aerial organs of *L. monopetalum* (leaves, stems, and flowers) were harvested in Ria de Alvor, Portimão (37° 07′ 34.7″ N, 8° 36′ 02.3″ W), in July 2020, whereas *C. mariscus* aerial parts (leaves and inflorescences) were collected in Ludo, Faro, southern Portugal (37° 01′ 03.3″ N, 7° 59′ 18.1″ W), in August 2020, in agreement with previous published phytochemical and anthelmintic data [13–15]. Plant harvesting licenses for the natural wild Portuguese territory were obtained, and standard procedures of “*Instituto da Conservação da Natureza e das Florestas (ICNF)*” were followed. Fresh biomass was washed, frozen, and freeze-dried (Lyofila 15, Telstar, Barcelona, Spain) for 3 days, transformed to powder using coffee and planetary ball mill (PM 100, Retsch, Haan, Germany), and stored in the dark till use.

2.1.2 Extraction of plant materials

Limoniastrum monopetalum (375 g) and *C. mariscus* (525 g) freeze-dried biomass was immersed in an 80% aqueous acetone solution (1:40, w/v), for 16 h, at 20–25 °C, in the dark and under magnetic stirring, aligned with the protocol formerly performed [13–15]. After fifteen solvent extractions were made for *L. monopetalum* and twenty-one for *C. mariscus*, each pooled extract was filtered and acetone removed using a rotary evaporator (reduced pressure and approx. 40 °C). Then, the acetone-free residue was freeze-dried, yielding approx. 75 g of dry extract for each species, later used for the phytochemical analysis and in vivo experiments.

2.1.3 Total phenolic (TPC) and condensed tannins (CTC) contents

The total phenolic content (TPC) of the extracts was assessed by the Folin–Ciocalteu method [17], while for the estimation of condensed tannins content (CTC) the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA–HCl) method [18] was performed, both methods using 96-well plates, as priorly detailed in [15].

2.1.4 Chemical characterization through HPLC-Q-TOF-MS

The chemical characterization was conducted similarly to that described in [13, 14], as detailed in the Supplementary material. Herein, the phytochemical analysis was conducted qualitatively, aiming at the evaluation of the main compounds present. Briefly produced extracts (5–10 mg) were resuspended in methanol and filtered before analysis using a Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 6530B quadrupole-time-of-flight mass spectrometer (Q-TOF MS), and a column Luna Omega Polar C18 of

150×3.0 mm and 5 µm particle size (Phenomenex, Torrance, CA, USA) with a Polar C18 Security Guard cartridge of 4×3.0 mm. The elution gradient, using water and formic acid 0.1% v/v (eluent A) and acetonitrile + formic acid 0.1% (eluent B), was as follows: 10–25% B in 0–25 min, 25% B in 25–30 min and 25–100% B in 30–35 min; in the end, eluent B was returned to 10% with a 7 min stabilization time, with a flow rate of 0.4 mL min^{−1}. The Q-TOF MS was operated in negative ion mode using an orthogonal ESI source (capillary voltage, 3500 V; nebulizer pressure of 45 psi; drying gas flow rate, 10 L/min; gas temperature, 325 °C; skimmer voltage, 60 V; fragmentor voltage, 140 V) and signals at m/z 112.9855 and 1033.9881 were used as internal calibration throughout analysis. Moreover, the MS and Auto MS/MS modes (collision energies of 10, 20 and 40 V) were set to acquire m/z values ranging between 50 and 1200, at a scan rate of 2 and 3 spectra per second, respectively, and the Agilent Mass Hunter Qualitative analysis software version B.06.00 was used for post-acquisition data processing.

2.2 Experimental design of the in vivo study

2.2.1 Production of infective larvae

Infective L3 larvae from anthelmintic-susceptible isolates of *Haemonchus contortus* or *Trichostrongylus colubriformis* were recovered by feces from monospecifically infected donor sheep using the Baermann technique, and at 4 °C until use in a single combined dose for the experimental infection of the lambs, as detailed in the next sub-section.

2.2.2 Animals, infection and treatment

Twenty Tarascon breed female lambs, between 3 and 4 months old, weighing 30–35 kg, were divided in three homogenous groups: a control non-treated group (C; n=6), a group receiving the *C. mariscus* extract (CM; n=7) and a group receiving the *L. monopetalum* extract (LM; n=7). Animals were maintained indoors, in experimental facilities with concrete floors and separated boxes (12 m² each), and water, hay, and mineral blocks were provided ad libitum.

The complete experimental design described herein is illustrated in Fig. 1. Lambs were kept in helminth-free conditions, testing negative for strongyle nematode infections seven days before infection (Fig. 1), using the McMaster technique [19]. To prevent coccidian infections and preserve animal welfare, diclazuril (Vecoxan, 2.5 mg mL^{−1}, Lilly, France) at 1 mg kg^{−1} of live weight was used twice at three weekly intervals. The study lasted for 7 weeks, starting from infection (Day 0=DPI 0). At this date, an oral administration of a single combined dose of 2000 L3 larvae of *H. contortus* and 500 L3 larvae of *T.*

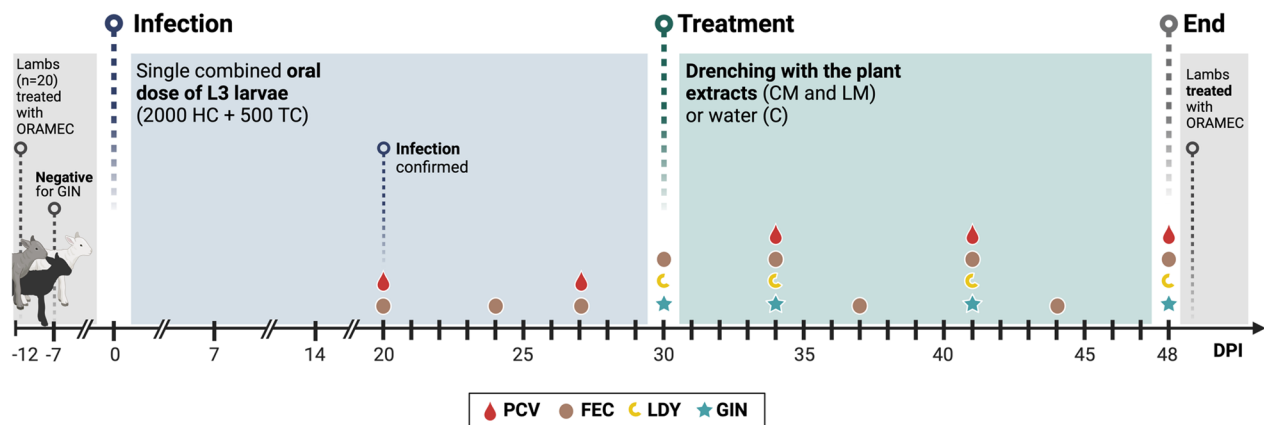


Fig. 1 Experimental design of the study. Pathophysiological and parasitological assessments are indicated beneath the timeline, with symbols located above days post-infection (DPI), representing that sampling was performed. PCV, packed cell volume; FEC, fecal egg counts; LDY, L3 larvae development yields; GIN, relative proportions between GIN species. Created in BioRender. Santos, F. (2025)

colubriformis in 20 mL of water. Twenty-one days post-infection (DPI 21), GIN infection was confirmed in the 3 experimental groups by fecal examination and individual measurements of eggs per gram (EPG). On DPI 30, lambs of groups CM and LM received individually 9.5 g of extract (0.3 g kg^{-1}), in 20 mL of water *per os*, while the control group was drenched with 20 mL of distilled water.

2.2.3 Infection follow-up

Individual blood samples were collected weekly (DPI 0, 7, 14, 21, 27, 34, 41, and 48) via jugular venipuncture to assess packed cell volume (PCV) levels, using a microhematocrit centrifuge and reader, an indicator of the pathophysiological effects across the three experimental groups. Parasitological evaluations were conducted twice a week following the confirmation of infection (DPI 21, 24, 27, 31, 34, 37, 41, 44, and 48). For fecal egg counts (FEC), individual fecal samples were obtained through a modified McMaster method as described by Raynaud (1970), and results expressed as eggs *per* gram of feces (EPG). Additionally, to evaluate the potential of the plant extracts on the parasite's life cycle, the development of eggs into infective L3 larvae of the two GIN species and as well as relative proportions of the two GIN species, were evaluated on DPI 30, 34, 41, and 48. On each evaluation date, after individual FEC, fecal samples *per* lamb were pooled in a group fecal culture. Three FEC were performed on these pooled groups of feces and the mean EPG was calculated for each pooled group sample. The total number of eggs in the pooled fecal sample was evaluated as (total weight of feces x mean number of eggs per gram).

The pooled fecal culture of each group (C, CM, and LM) was maintained in similar conditions, and after

10 days, L3 larvae were collected, according to the Baermann method.

The total number of infective L3 larvae was evaluated, and the yield L3 development from eggs was given by the following formula:

$$\text{Number of eggs in the fecal sample} = \frac{\text{Number of L3 collected after 10 days larva cultures}}{\text{weight of feces} \times \text{number of eggs per gram}}$$

In addition, after L3 larvae collection, the identification of the infective larvae species and their relative proportions was estimated on 100 L3 larvae, on each date and for each group [20].

2.2.4 Ethical considerations

The *in vivo* trial was conducted at the facilities of the National Veterinary School of Toulouse (ENVT) in southwest France ($43^{\circ} 35' 59'' \text{ N}$, $1^{\circ} 22' 41'' \text{ E}$). Animal handling and care, as well as the trial protocol design, were carried out in compliance with the French ethical and welfare regulations and approved by the Comité d'Éthique en Expérimentation Animale (Science et Santé Animales, SSA N° 115, December 15, 2014).

2.3 Statistical analyses

Before analyses, FEC data were normalized by using a transformation $\text{Log}(n+1)$, as normality was not assumed for EPG, while PCV data were normally distributed. Using the SYSTAT software, FEC and PCV results have been analyzed first date-by-date, using one-way ANOVA, on each date post-infection (DPI; 0 to 48) for PCV, and DPI 21, DPI 24, 27, 31, 34, 37, 41, 44, 48, for FEC. In a second step, the PCV and FEC data obtained from DPI 21 to DPI 48 were analyzed through repeated measurements, considering "treatment" and "time." For technical

reasons, the development of eggs to L3 larvae has been applied on pooled fecal samples *per* experimental group on different dates, therefore, restraining further statistical analyses on the development yield and on the species composition of larvae.

3 Results

The extraction yields obtained were $21.9 \pm 0.9\%$, for *L. monopetalum*, and $15.5 \pm 1.8\%$, for *C. mariscus*. Both total phenolic (TPC) and condensed tannins content (CTC) values were similar between plant species: in TPC, *C. mariscus* extract exhibited 243.2 mg GA eq. g⁻¹ extract DW while *L. monopetalum* showed 236.8 mg GA eq. g⁻¹ extract DW; in CTC, *C. mariscus* extract presented

132.3 mg CT eq. g⁻¹ extract DW while *L. monopetalum* had 124.0 mg CT eq. g⁻¹ extract DW.

Chemical characterization of the samples performed by HPLC–MS–Q–TOF analysis is detailed in Tables 1 and 2, along with the main parameters (exact mass, molecular formula, error and fragmentation), and the respective chromatographic profiles are illustrated in Fig. 2. Relative abundances (RA) were estimated *per* compound; however, comparisons should be cautious since ionization efficiencies differ between compounds.

C. mariscus extract was mainly comprised of luteolin C-glycosides (combined relative abundance (RA)=25%), flavan-3-ols (epigallocatechin, epicatechin, catechin; combined RA=15.15%), luteolin (RA=12.33%),

Table 1 Chemical characterization by HPLC-MS-Q-TOF of the compounds found in the extract of *Cladium mariscus*. A heat map (last column) was performed to determine the relative contribution of each compound to the whole extract (darker color corresponds to higher abundance). For each compound, the peak area in MS mode was determined using the precursor ion [M-H]⁻; then, relative contribution (%) of each compound regarding the sum of the areas of all compounds was calculated. **t_R**, retention time; **RA**, relative abundance (%)

No.	t _R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
1	1.8	377.088	C ₁₂ H ₂₂ O ₁₁	-4.97	341.1093, 179.0554, 161.0450, 143.0338, 119.0341, 113.0237, 101.0243, 89.0243	Disaccharide (HCl adduct)	11.55
3	2.4	133.0142	C ₄ H ₆ O ₅	0.23	115.0032, 71.0144	Malic acid	4.59
6	4.8	305.0671	C ₁₅ H ₁₄ O ₇	-1.57	261.0861, 219.0624, 179.0345, 125.0240	(Epi)gallocatechin	0.85
7	7.3	577.1361	C ₃₀ H ₂₆ O ₁₂	-0.76	451.1040, 425.0880, 305.0671, 289.0714	Proanthocyanidin dimer	1.27
8	7.7	305.0671	C ₁₅ H ₁₄ O ₇	-1.26	261.0661, 219.0643, 179.0345, 125.0239	(Epi)gallocatechin	9.03
10	9.2	289.0721	C ₁₅ H ₁₄ O ₆	-1.15	245.0829, 205.0524, 203.0657, 179.0344	Catechin	4.29
11	9.5	353.0885	C ₁₆ H ₁₈ O ₉	-1.67	191.0554, 179.0332, 173.0459	Chlorogenic acid	2.15
12	9.6	865.2065	C ₄₅ H ₃₈ O ₁₈	-0.78	739.1536, 713.1536, 695.1377, 577.1388, 451.0995, 425.0894	Proanthocyanidin trimer	0.15
13	10	429.1046	C ₁₈ H ₂₂ O ₁₂	-1.69	267.0718, 161.0236	Unknown	7.64
14	10.3	577.1352	C ₃₀ H ₂₆ O ₁₂	-0.75	451.1004, 425.0873, 305.0654, 289.0738	Proanthocyanidin dimer	0.83
15	10.5	865.2065	C ₄₅ H ₃₈ O ₁₈	-1.17	739.1612, 713.1534, 695.1387, 577.1399, 451.1191, 425.0911	Proanthocyanidin trimer	0.11
16	10.5	561.1413	C ₃₀ H ₂₆ O ₁₁	-0.51	447.0908, 435.1088, 409.0961, 289.0762	Proanthocyanidin dimer	1.36
18	11.3	577.1358	C ₃₀ H ₂₆ O ₁₂	-1.43	451.1094, 425.0873, 305.0656, 289.0708	Proanthocyanidin dimer	0.24
19	12	577.135	C ₃₀ H ₂₆ O ₁₂	-0.05	451.1043, 425.0881, 305.0656, 289.0725	Proanthocyanidin dimer	1.51
20	12.1	289.0727	C ₁₅ H ₁₄ O ₆	-2.93	245.0808, 205.0491, 203.0708, 179.0353	Epicatechin	0.98
21	12.9	579.1362	C ₂₆ H ₂₈ O ₁₅	-0.8	561.1214, 519.1120, 489.1051, 459.0929, 399.0703, 369.0628	Luteolin-C-glucosyl-O-arabinoside	0.32
23	14.3	579.1362	C ₂₆ H ₂₈ O ₁₅	-1.5	561.1212, 519.1120, 489.1036, 459.0925, 399.0737, 369.0596	Luteolin-C-glucosyl-O-arabinoside	9.09
25	14.9	865.2065	C ₄₅ H ₃₈ O ₁₈	0.7	739.1603, 713.1553, 695.1410, 577.1330, 451.1068, 425.0901	Proanthocyanidin trimer	0.29
27	16.4	563.1414	C ₂₆ H ₂₈ O ₁₄	-1.44	545.1211, 503.1223, 473.1103, 443.0988, 413.0901, 383.0787, 353.0657	Apigenin-C-glucoside-C-arabinoside	2.06
28	16.6	163.0399	C ₉ H ₈ O ₃	0.93	119.0497	Coumaric acid	1.34
29	17.3	447.0941	C ₂₁ H ₂₀ O ₁₁	-1.76	429.0823, 357.0615, 327.0512, 285.0401	Luteolin-6-C-glucoside (isoorientin)	10.22
30	17.3	549.1257	C ₂₅ H ₂₆ O ₁₄	-0.91	531.1177, 489.1017, 459.0932, 441.0885, 399.0733, 369.0474	Luteolin-di-C-arabinoside	2.3
31	17.8	461.1095	C ₂₂ H ₂₂ O ₁₁	-1.29	341.0653, 327.0506, 313.0363, 298.0483	Unknown	3.67
32	18.2	563.1409	C ₂₆ H ₂₈ O ₁₄	-1.52	545.1276, 473.1073, 443.0949, 413.0949, 383.0794, 353.0654	Apigenin-C-glucoside-C-arabinoside	0.2
34	19.9	533.1297	C ₂₅ H ₂₆ O ₁₃	0.48	515.1251, 473.0984, 443.0957, 413.0857, 383.0788, 353.0646	Apigenin-di-C-pentoside	0.28
35	20.1	303.0516	C ₁₅ H ₁₂ O ₇	-2.27	285.0410, 125.0232	Taxifolin (dihydroquercetin)	0.17
36	20.6	431.0988	C ₂₁ H ₂₀ O ₁₀	-1.14	413.0889, 383.0724, 341.0669, 311.0562, 283.0620	Apigenin-C-hexoside	1.26
39	21.9	447.0945	C ₂₁ H ₂₀ O ₁₁	-3.24	285.0387	Kaempferol-O-hexoside	2.87
40	22.5	417.0835	C ₂₀ H ₁₈ O ₁₀	-1.76	399.0740, 357.0614, 327.0523, 297.0361, 285.0396	Luteolin-C-pentoside	3.07
41	22.7	243.0662	C ₁₄ H ₁₂ O ₄	-0.04	201.0549, 159.0436	Unknown	2.5
45	27	317.0308	C ₁₅ H ₁₀ O ₈	-1.83	271.0177, 178.9974, 151.0030	Myricetin	1.46
47	36.1	285.041	C ₁₅ H ₉ O ₆	-1.88	---	Luteolin	12.33

Table 2 Chemical characterization by HPLC-MS-Q-TOF of the compounds found in the extract of *Limoniastrum monopetalum*. A heat map (last column) was performed to determine the relative contribution of each compound to the whole extract (darker color corresponds to higher abundance). For each compound, the peak area in MS mode was determined using the precursor ion $[M-H]^-$; then, the relative contribution (%) of each compound regarding the sum of the areas of all compounds was calculated. t_R , retention time; **RA**, relative abundance (%)

No.	t_R (min)	Observed [M-H] ⁻	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
1	1.8	377.0868	C ₁₂ H ₂₂ O ₁₁	-2.93	341.1085 , 179.0559, 161.0326, 143.0328, 119.0342, 113.0236, 101.0238, 89.0243	Disaccharide (HCl adduct)	1.58
2	1.9	191.0199	C ₆ H ₈ O ₇	-1.01	173.0084, 111.0083 , 87.0091	Isocitric acid	1.72
3	2.4	133.0142	C ₄ H ₆ O ₅	0.4	115.0025 , 71.0149	Malic acid	0.32
4	2.5	191.0199	C ₆ H ₈ O ₇	-0.73	173.0088, 111.0089 , 87.0091	Citric acid	0.44
5	3.3	169.014	C ₇ H ₆ O ₅	1.27	125.024	Gallic acid	1.7
6	4.8	305.0669	C ₁₅ H ₁₄ O ₇	-0.59	261.0757, 219.0674, 179.0352, 125.0237	(Epi)gallocatechin	0.97
8	7.7	305.0672	C ₁₅ H ₁₄ O ₇	-1.64	261.0800, 219.0670, 179.0354, 125.0246	(Epi)gallocatechin	0.66
9	9.1	303.0187	C ₁₁ H ₁₂ O ₈ S	-2.29	223.0605 , 208.0370, 179.0694, 164.0480, 149.0240	Sinapic acid sulfate	1.82
17	10.6	273.0079	C ₁₀ H ₁₀ O ₇ S	-1.82	193.0501 , 178.0267, 149.0593, 134.0366	Ferulic acid sulfate	8.17
22	13.9	457.1165	C ₂₀ H ₂₆ O ₁₀ S	1.45	329.1398, 169.0134 , 125.0237	Gallic acid derivative	3.14
24	14.3	457.1181	C ₂₀ H ₂₆ O ₁₀ S	-1.55	329.1397 , 245.0123, 195.0654, 165.0549	Unknown	2.05
26	15.6	411.0032	C ₁₆ H ₁₂ O ₁₁ S	-0.55	331.0459 , 271.0161	Unknown (sulfate adduct)	0.51
33	19.8	425.019	C ₁₇ H ₁₄ O ₁₁ S	-0.92	345.0615 , 330.0375, 315.0178	Dimethylated flavonoid (sulfate adduct)	0.6
37	20.7	463.0893	C ₂₁ H ₂₀ O ₁₂	-2.19	301.0399 , 151.0393	Quercetin-O-hexoside	0.8
38	21.7	477.068	C ₂₁ H ₁₈ O ₁₃	-1.05	301.0353 , 178.9994, 151.0026	Quercetin-O-glucuronide	1.02
42	23.7	437.0918	C ₂₀ H ₂₂ O ₉ S	-1.5	357.1350, 151.0394 , 136.0162	Pinoresinol sulfate	4.09
43	24.8	395.0088	C ₁₆ H ₁₂ O ₁₀ S	-2.46	315.0511 , 300.0293, 271.0254	Isorhamnetin sulfate	13.83
44	25.6	425.0197	C ₁₇ H ₁₄ O ₁₁ S	-2.93	345.0620 , 330.0383, 315.0150, 271.0262, 287.0195	Methylated flavonoid (sulfate adduct)	25.93
45	27	317.0309	C ₁₅ H ₁₀ O ₈	-1.99	178.9978, 151.0029	Myricetin	0.23
46	32.2	439.0357	C ₁₈ H ₁₆ O ₁₁ S	-3.4	359.0775 , 344.05413, 329.0299	Methylated flavonoid (sulfate adduct)	28.61
48	38.6	327.2182	C ₁₈ H ₃₂ O ₅	-1.1	291.1949, 229.1447, 211.1333 , 171.1013	Oxo-dihydroxyoctadecenoic acid	0.93
49	40.2	329.2331	C ₁₈ H ₃₄ O ₅	1.2	311.2071, 283.2588, 229.1390, 211.1339 , 171.1015	Trihydroxy-octadecenoic acid	0.9

proanthocyanidins (combined RA=5.76%) and apigenin C-glycosides (RA=3.8%), accounting for 62% of the extract relative composition. Some compounds remain to be identified (**13**, **31**, **41**; combined RA=13.81%) but malic acid (**3**), coumaric acid (**28**), taxifolin (**35**) have been herein identified for the first time in *C. mariscus* extract.

The highest contributors in the *L. monopetalum* extract (RA=54.54%) were the methylated flavonoids sulfate **44** and **46**, followed by isorhamnetin sulfate (**43**, RA=13.83%), ferulic acid sulfate (**17**, RA=8.17%), pinoresinol sulfate (**42**, RA=4.09%) and a gallic acid derivative (**22**, RA=3.14%), in line with the previous chemical characterization performed [13]. Minor compounds such as isocitric (**2**), malic (**3**) and citric (**4**) acids, quercetin-O-hexoside (**37**) and myricetin (**45**) were not detected in *L. monopetalum* extract before [13].

To have an overview of the potential of the extracts on infected animals, the experimental protocol was designed to assess its anthelmintic value in two frontlines: (1) consequences on lamb resilience and (2) effects on the biology of parasite populations in lambs either on the worm populations in the host or on the egg development to L3 coupled with GIN proportions in mixed infections, following the conceptual frame proposed in [10].

Regarding pathophysiological measurements (Fig. 3), independently of the experimental group, no signs of anemia were observed, and packed cell volume (PCV) values remained within normal values (30–40%) among groups. PCV measures the proportion of blood occupied by erythrocytes; thus, its decline is a common indicator of anemia, which is particularly relevant to monitor *H. contortus* infections, due to the species' hematophagous behavior. Overall, the comparison between groups by two-way ANOVA, based on repeated measurements, on DPI 34, 41, and 48 data, revealed statistical differences ($p < 0.05$). Results of the statistical date-by-date analyses, based on one-way ANOVA from DPI 30, indicated significant differences on DPI 34, DPI 41 ($p < 0.05$) and DPI 48 ($p < 0.01$).

A first parasitological evaluation of the consequences of administration of the two plant extracts relied on FEC (Fig. 3), frequently used to assess GIN infection levels in small ruminants and estimations of future pasture contamination. In this work, no proven in vivo anthelmintic effects, at a dose of 0.3 g kg⁻¹, were verified for the two plant extracts as no statistical differences were observed between experimental groups, either based on the repeated measurements analyses (DPI 30–48) or by referring to the date-by-date analyses. Lastly, when a

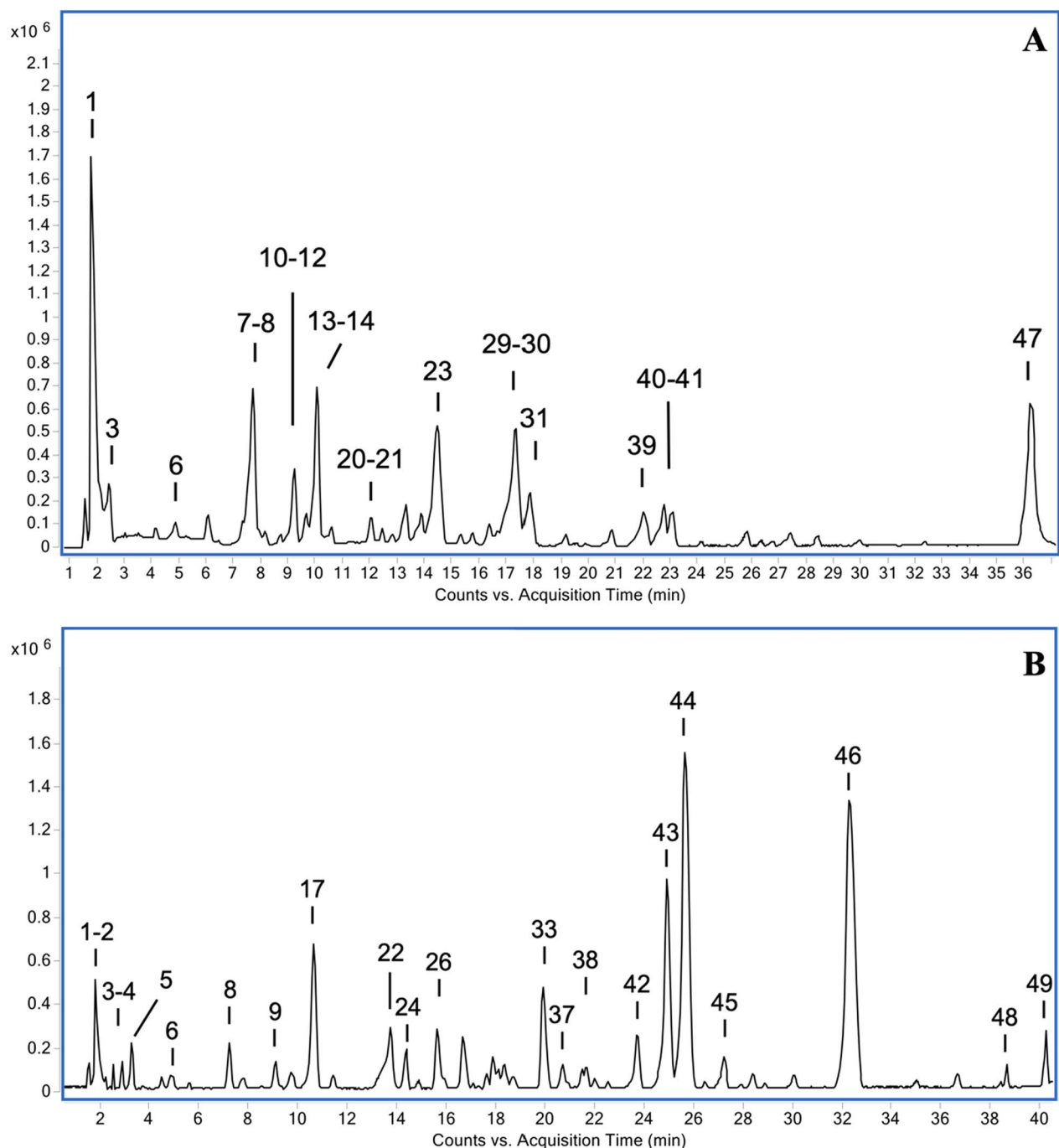


Fig. 2 HPLC-ESI-Q-TOF-MS base peak chromatograms of *C. mariscus* (A) and *L. monopetalum* (B) 80% acetone:water extracts

co-variance analysis was applied date-by-date, using log values of EPG ($n + 1$) on DPI 30 as a co-variable, no significant differences were observed on each date up to DPI 48.

Yields of egg development to L3 larvae and GIN species proportions were evaluated on pooled fecal cultures by groups and results are presented in Table 3. Although variations were observed over time in larvae yields, results were mostly homogeneous between groups, and main trends were not observed in the experimentally treated groups when compared to the

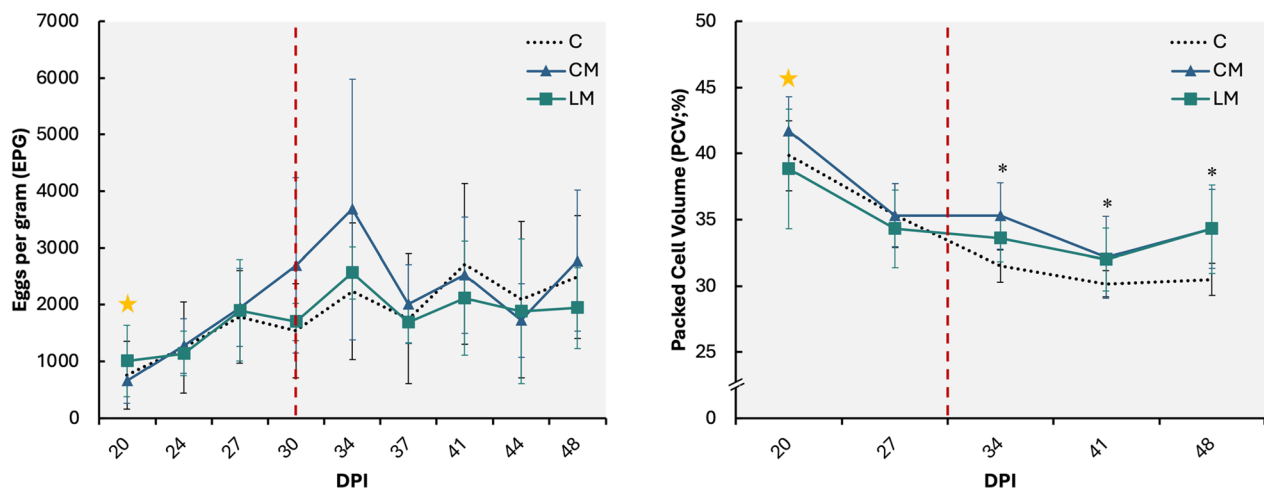


Fig. 3 Mean EPG and PCV values and respective standard deviations in the three experimental groups in study. C, control group; CM, group receiving *C. mariscus* extract; LM, group receiving *L. monopetalum* extract. Lambs' infections were confirmed on DPI 20 (yellow star), and drenching with the plant extracts was performed on DPI 30 (red dashed vertical line). * represents statistical differences between groups, using two-way ANOVA based on repeated measurements

Table 3 L3 larvae developed from eggs (%) and relative proportions (%) of *H. contortus* in relation to *T. colubriformis* L3 larvae evaluated in the three experimental groups in study: C, control group; CM, group receiving *C. mariscus* extract; LM, group receiving *L. monopetalum* extract. Lambs' infections were confirmed on DPI 20 and drenching with the plant extracts was performed on DPI 30

Group/DPI	30	34	41	48
<i>L3 larvae development yields (%)</i>				
C	7.05	11.4	9.89	8.72
CM	12.2	7.94	8.2	13.4
LM	3.85	13.4	8.41	8.5
<i>Relative proportions (%) of H. contortus L3 larvae</i>				
C	95	97.7	100	99
CM	95	88	95.4	98
LM	94	89.6	98	98

control. Concerning GIN species proportions, regardless of the timepoint considered, *H. contortus* larvae were consistently dominant, ranging from 86 to 100%. Having in mind the initial infection ratio (2000 L3 larvae of *H. contortus* to 500 of *T. colubriformis*) and the non-significant results obtained concerning worm biology (FEC values), these GIN proportion results are expected considering that *H. contortus* is a highly prolific egg producing parasite (daily egg output range from 5.000 to 15.000 eggs per female) while *Trichostrongylus* spp. female nematodes may expel daily only a few hundred [21, 22].

4 Discussion

Despite a panoply of studies demonstrating the in vitro effects of plant extracts/or fractions against different life processes of *H. contortus* and *T. colubriformis* [23, 24], there is a paucity of investigations validating the anthelmintic value of salt-tolerant plant crude extracts in small ruminant hosts, through in vivo trials. In agreement, this work was designed to address this issue by looking for the first proof of concept of the in vivo anthelmintic significance of hit salt-tolerant plant extracts.

Since phenolic composition can vary across harvesting periods [15], the first goal was to phytochemically characterize the produced extracts to corroborate chemical data from previous in vitro assessments [13]. In previous studies, TPC levels were higher than those recorded herein and *L. monopetalum* collected in July 2017 had increased CTC [13, 15]. Given that both species were collected from the same location and season and processed using similar methodological settings, variations in phenolic contents may result from plant species-specific adjustments in the wild, through chemical defenses in response to environmental conditions in each harvesting year (2017 vs 2020). Nonetheless, the chromatographic profiles were comparable to those obtained earlier by HPLC-ESI-MS [13, 14]. Herein, the use of HPLC-Q-TOF-MS offers an additional advantage of high-resolution mass spectrometry, allowing the confirmation of compound identities through molecular formulas.

Although major metabolites such as flavan-3-ols, proanthocyanidins and luteolin have been previously identified as bioactive anthelmintic molecules [25–27], the potential contribution of minor metabolites should

not be overlooked, as they may influence activity in a synergistic or antagonistic manner. As an example, myricetin derivatives have been found before in plant extracts with strong anthelmintic effects [28], while taxifolin was ineffective in inhibiting larvae exsheathment [26]. Indeed, Olmedo-Juaréz and colleagues (2022) inferred that when kaempferol was individually tested did not present ovicidal activity on *H. contortus* eggs, but in the presence of minor quantities of ferulic acid, the mixture was highly effective [29].

In the end, the phytochemical characterization of the two plant extracts was consistent with previous in vitro results [13], supporting the interest in testing these extracts' in vivo anthelmintic effects, as a detailed chemical characterization is fundamental whenever aiming at the development of novel standardized phytotherapeutic products against GIN.

Under the tested in vivo experimental conditions, mostly non-significant parasitological effects were registered with the applied single dose. However, a significant positive effect of the extracts on lambs' hematopoiesis, noted through higher PCV values in these groups, was observed. PCV is usually negatively correlated to FEC values, and both parameters serve as phenotypic markers of sheep resilience to GIN infections [30]. Although significant changes in FEC values were not observed, differences in PCV levels in relation to the control group raise the hypothesis that some bioactive plant extracts might stimulate lambs' erythropoietic system and overall animal immune status. As proven, both extracts are rich sources of a range of phenolic metabolites, well-known for their antioxidant capacity. Parasite infections trigger oxidative stress in ruminant animals [31, 32], which can damage erythrocytes, increase hemolysis rate and, ultimately, contribute to anemia [33]. Fang and colleagues (2016) proved that the flavonoids orientin and luteolin can attenuate the hemolysis rate of erythrocytes under oxidative stress, preserving their cell structural integrity and, consequently, prolonging their lifespan [33]. In addition, the inclusion of polyphenol-rich plants in the diet of infected lambs has been shown to slow the infection dynamics, improving the host's overall antioxidant status and resistance to infection [34, 35]. Although it is plausible that these extracts can boost the animal's overall health status, further research is needed to corroborate this assumption.

The absence of significant parasitological findings in in vivo trials can be interpreted in two ways: either the plant extracts are inherently ineffective, or the experimental design requires adjustments, e.g., dose, frequency of administration, and duration of the study. Drawing clear conclusions is challenging due to the difficulty of transposing in vitro results to in vivo conditions. Indeed,

plant-derived products often show better efficacy in vitro, likely due to changes in bioavailability and biotransformation of active metabolites throughout the ruminant digestive system, as well as potential synergistic/antagonistic interactions between compounds [36–38], underscoring the importance of a detailed phytochemical assessment.

While established protocols exist for in vitro screening of plant extracts [39], recommendations for validating bioactive nutraceutical plants [10], and guidelines for evaluating synthetic anthelmintics [40, 41], there is still a lack of well-defined guidelines for in vivo trials involving plant-based extracts. Consequently, as highlighted in [24], despite a growing number of in vivo anthelmintic studies using plant extracts with confirmed bioactivity, applied methodologies vary considerably, limiting direct comparisons and definitive conclusions [24]. Among major methodological challenges are the extracts' mode of preparation, dosage, type, and duration of treatment (e.g., single vs. multiple doses, short-term drenches vs. long-term feed incorporation) [24].

Herein, the single dose applied does not establish the absence or presence of anthelmintic effects of the two plant extracts in vivo experimentally infected lambs; therefore, it cannot be ruled out that higher doses and multiple administrations might produce measurable outcomes. Comparably, other works also struggled to translate promising in vitro to in vivo results. For example, Oliveira and colleagues (2009) found that the ethyl acetate extract obtained from liquid of green coconut husk fiber did not present in vivo effects in naturally infected sheep (0.4 g kg⁻¹; three consecutive doses), although being active on in vitro egg and larvae assays against *H. contortus* [42]. Egualé and colleagues (2007) tested two single doses of *Coriandrum sativum* aqueous extracts (0.45 and 0.9 g kg⁻¹), which were highly active in vitro on eggs and adult worms, against *H. contortus* experimentally infected sheep; however, discreet significant results on egg excretion and worm burden were observed, mostly in the higher dose used, while PCV values were not improved with both doses [43]. Cala et al. (2014) observed that a single dose of artemisinin (100 mg kg⁻¹) and its plant source, *Artemisia annua* aqueous extract (2 g kg⁻¹), were ineffective in sheep naturally infected with GIN [44]. The authors argued that plant-derived products should be tested in multiple doses, particularly when animals have high EPG counts, and underline that the benefits of using plant extracts could be potentially greater in combination with synthetic drugs [44].

Elsewhere, more encouraging results have also been obtained. Lone et al. (2012) demonstrated an egg count reduction with a single dose of 86% and 44% (1 g kg⁻¹) methanolic and aqueous extracts of *Euphorbia*

helioscopia, respectively, after 18 days of post-treatment [45]. Similarly, the crude methanol extracts of *Acacia nilotica* bark and leaves resulted in significant reductions in fecal egg counts (FECR; 63–72%, respectively, at 8 g kg⁻¹, 12 days post-treatment) [46], while a single dose (3.0 g kg⁻¹) of the methanolic extract of its fruits had a FECR of 78.5% in infected sheep [47]. Ethanolic and aqueous extracts of the aerial organs of *A. absinthium*, tested at 1 and 2 g kg⁻¹ (single doses) were effective in reducing egg counts of naturally infected sheep, particularly the ethanolic extract at 2 g kg⁻¹ (FECR of 90%, day 15 post-treatment) [48]. Recent research also reported high efficacy of hydroethanolic leaf extract of *Combretum mucronatum* in goats infected with *H. contortus*, achieving up to 85.9% FECR even at lower doses (0.25 mg kg⁻¹; four consecutive days) [49].

Still, according to the WAAVP guidelines [40, 41], an anthelmintic is highly effective if a minimum of 90% reduction in parasite counts is recorded. As seen above, plant-based products often fall short of this threshold, typically showing lower efficacy than synthetic drugs [24, 50]. As suggested by Githiori and colleagues [50], to improve the validation and inclusion of herbal products in integrated parasite control strategies for ruminant farming systems, it may be beneficial to revise this efficacy threshold, alongside standardized experimental designs.

5 Conclusions

This work represents the first attempt to evaluate the *in vivo* anthelmintic value of salt-tolerant species-derived extracts, namely *C. mariscus* and *L. monopetalum*. The validation of the phytochemical profiles of these extracts, as compared to previous *in vitro* studies, is of utmost importance for understanding the observed biological effects and for the future standardization of potential phytotherapeutic products.

Overall, at the dose tested, the plant extracts did not unveil the absence or presence of significant anthelmintic outcomes, despite a registered significant increase in PCV values, raising the hypothesis of a potential improvement in the overall health status of the animals. Future work should focus on testing higher and/or multiple doses of the plant extracts, as well as investigating their bioavailability, which may significantly enhance the *in vivo* anthelmintic value of these phytotherapeutic products. The lack of established protocols for evaluating the anthelmintic efficacy of plant extracts limits the comparison and interpretation of existing literature, yet gathering *in vivo* evidence remains crucial to pave the way toward the development of novel plant-based solutions to fight GIN infections.

Abbreviations

C	Control group
CM	Group receiving <i>Cladium mariscus</i> extract
LM	Group receiving <i>Limonium monopetalum</i> extract
DPI	Days post-infection
PCV	Packed Cell Volume
GIN	Gastrointestinal nematodes
L3	Third-stage larvae
ICNF	Instituto da Conservação da Natureza e das Florestas
TPC	Total Phenolic Content
CTC	Condensed Tannins Content
DMACA-HCl	4-dimethylaminocinnamaldehyde-hydrochloric acid
Q-TOF MS	Quadrupole-Time-Of-Flight Mass Spectrometer
EPG	Eggs per gram
FEC	Fecal Egg Counts
ENVT	National Veterinary School of Toulouse
RA	Relative Abundance
HPLC-ESI-MS	High-Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry
FECR	Fecal Egg Count Reduction
WAAVP	World Association for the Advancement of Veterinary Parasitology

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-025-00661-7>.

Additional file1 (DOCX 20 KB)

Author contribution

MO: conceptualization, investigation, writing—original draft; AD, LM, EB, and EP: investigation; EL-M: investigation, review and editing; HH: conceptualization, formal analysis, supervision, validation; KBH: funding acquisition, project administration; LC: conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, writing—review and editing.

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Data availability

Data will be made available upon request.

Declarations

Conflict of interests

The authors declare no competing interests.

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