

In vitro anthelmintic activity of active compounds of the fringed rue *Ruta chalepensis* against dairy ewe gastrointestinal nematodes

E. Ortu¹, G. Sanna², A. Scala², G. Pulina¹, P. Caboni^{3*} and G. Battacone¹

¹Department of Agriculture, University of Sassari, via Enrico de Nicola 7, 07100 Sassari, Italy; ²Department of Veterinary Medicine, University of Sassari, via Vienna 2, 07100 Sassari, Italy; ³Department of Life and Environmental Sciences, University of Cagliari, via Ospedale 72, 09124 Cagliari, Italy

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Abstract

Infections by gastrointestinal nematodes negatively affect small ruminant health and at the same time cause substantial economic losses worldwide. Because resistance to conventional anthelmintic compounds is growing, target studies evaluating the effectiveness of alternative ingredients of botanical origin on gastrointestinal nematodes are needed. In this study, we evaluated the *in vitro* anthelmintic activity of *Ruta chalepensis* L. extracts on the third-stage larvae of sheep gastrointestinal nematodes. A methanol extract showed the highest anthelmintic activity, with an $EC_{50} = 0.10 \pm 0.06$ mg/ml after 96 h, while the essential oil had an $EC_{50} = 1.45 \pm 1.22$ mg/ml after 48 h. Moreover, three secondary metabolites of the essential oil, i.e. 2-decanone, 2-nonanone and 2-undecanone, showed EC_{50} values of 0.07 ± 0.06 , 0.25 ± 0.29 and 0.88 ± 0.73 mg/ml at 24 h, respectively. The present study indicated that the *R. chalepensis* methanol extract, the essential oil and its metabolites 2-decanone, 2-nonanone and 2-undecanone showed promising anthelmintic activity on gastrointestinal nematodes.

Introduction

Infections by gastrointestinal nematodes (GIN) influence sheep and goat health and their welfare status dramatically, limiting productivity by removing nutrients from the ingesta and producing indirect effects on the immune response (Sargison, 2012). Anthelmintic drugs in association with other strategies, such as grazing management, genetic selection and nutritional manipulation, are essential for the control of GIN infestations (Sargison, 2008; Sutherland & Scott, 2010; Hoste & Torres-Acosta, 2011). In particular, strategies of grazing management

aim to decrease the timing and extent of parasite risk by dispersion of larval density and by accelerating larval mortality (Torres-Acosta & Hoste, 2008). In recent years, interest in the use of forages and plants with anthelmintic properties in the diets of small ruminants has increased (Hoste & Torres-Acosta, 2011). Plants are considered a valid alternative to conventional anthelmintic drugs (Hammond *et al.*, 1997, Torres-Acosta & Hoste, 2008, Hoste & Torres-Acosta, 2011). In particular, studies have been conducted to evaluate the anthelmintic activity of the Meliaceae family, such as *Melia azedarach* L. Szewczuk *et al.* (2006) reported that the drupe extracts of this plant showed anthelmintic activity against *Bunostomum trigonocephalum* and *Oesophagostomum columbianum*. Moreover, the seed ethanol extract of *M. azedarach* revealed activity against *Haemonchus contortus* eggs, while in a larval

*Fax: 0039 070 6758612
E-mail: caboni@unica.it

development test the leaf ethanol extract showed an $LC_{50} = 9.18$ mg/ml (Maciel *et al.*, 2006). Currently, the condensed tannins contained in tannin-rich fodders are the most studied compounds with anthelmintic activity in small ruminants infected by GIN (Hoste & Torres-Acosta, 2011). Molan *et al.* (2003) studied the *in vitro* anthelmintic effects of monomer units of condensed tannins called 3-flavan-3-ols and their galloyl derivatives on *Trichostrongylus colubriformis*, finding that epigallocatechin gallate was more active than the other 3-flavan-3-ols in an egg hatch assay.

Ntalli *et al.* (2011) reported the nematicidal activity of the essential oil and the methanol extract of *Ruta chalepensis* L. on root knot nematodes. *Ruta chalepensis* essential oil was more active if compared with the methanol extract, and 2-undecanone was considered to be the main component responsible for the nematicidal effect. In addition, essential oils of fruits and leaves showed inhibition effects on *H. contortus* egg hatching (Hussien *et al.*, 2011) and adult worm motility (Akkari *et al.*, 2015).

The aim of the present study was to evaluate the *in vitro* anthelmintic activity of *R. chalepensis* methanol extract (RME), the essential oil (REO) and its metabolites 2-nonanone, 2-undecanone and 2-decanone. In addition, their anthelmintic activity was compared with that of other phytochemicals, such as furfural, salicylaldehyde, (*E,E*)-2-4-decadienal and epigallocatechin gallate.

Materials and methods

Chemicals and plant materials

Standards of 2-undecanone (UND), 2-nonanone (NON), 2-decanone (DEC), (*E,E*)-2-4-decadienal (DDI), furfural (FUR), salicylaldehyde (SAL), epigallocatechin gallate (ECG), gallic acid (GAA) and levamisole hydrochloride (LEV) of purity greater than 98%, as well as Tween 20, sodium phosphate dibasic, sodium phosphate monobasic and methanol were purchased from Sigma-Aldrich (Milano, Italy). The methanol, ethanol and water used were high-performance liquid chromatography (HPLC) grade.

Aerial parts of *R. chalepensis* were collected in February 2014 at Cagliari, Italy and were dried in the absence of light at room temperature in paper bags for 30 days. Botanical identity of the plants was verified and they were deposited in the Department of Life and Environmental Sciences, University of Cagliari, Italy. Dried aerial plant parts (100 g) were ground and extracted with methanol (1:10 w/v). After 20 h samples were homogenized with a sonicator for 15 min, filtered through a Whatman 40 filter and centrifuged for 15 min at 13,000 rpm, according to Ntalli *et al.* (2011). Aerial parts of *R. chalepensis* were subjected to hydro-distillation for 4 h. Essential oil was extracted in hexane and was dried over anhydrous magnesium sulphate. After filtration, the hexane was evaporated at low temperature under low pressure. The essential oil obtained and the methanol extract were kept in dark glass vials at -20°C until use. Yields of the essential oil and the extract were determined on average over four replicates.

GC-MS analysis

The essential oil was diluted (1:200 v/v) with hexane and injected for gas chromatography-mass spectrometry (GC-MS) analysis according to Ntalli *et al.*, (2011) and Caboni *et al.* (2012) with slight modifications. The chromatographic separation and identification of the main components were performed on a Trace GC ultra gas chromatograph (Thermo Finnigan, San Jose, California, USA) coupled with a Trace DSQ mass spectrometry detector, a split-splitless injector and an Xcalibur MS platform. The column used was polar fused silica capillary DB-5MS (5% phenylmethylpolysiloxane, 30 m \times 0.25 mm; film thickness, J&W 0.25 μm ; Scientific Fisons, Folsom, California, USA). The injector and transfer line were at 250°C , while the oven operating conditions were as follows: from 50°C (held for 5 min) to 280°C at $9^{\circ}\text{C}/\text{min}$ heating rate, and kept at this temperature for 8 min. The carrier gas was helium with a constant flow rate of 1 ml/min; the sample (0.20 μl) was injected in splitless mode (60 s). Mass spectrometry acquisition was carried out using the following conditions: ionization voltage, 70 eV; scan rate 1.6 scan/s; mass range, 50–550 amu. Essential oil components were identified by comparison of their retention times and mass fragmentation with those of authentic standards and computer matching against a NIST98 commercial library, as well as Kovats retention indices, for alkanes C9–C24 compared with those reported by Adams (2007). Moreover, concentrations of essential oil components were expressed as area percent.

In vitro assays

Faecal samples were collected from the rectum of Sarda dairy ewes and kept at 4°C until use. Third-stage larvae (L3) of GIN were obtained by coproculture: briefly, the faeces were broken up and placed into a glass jar, which was closed and kept at a temperature of about 27°C for 14 days. After that, larvae were separated following the Baermann technique (Euzeby, 1981) and identified using the morphological keys by MAFF (1986). Larvae were identified as belonging to the following GIN species: *Teladorsagia* spp. (52%), *H. contortus* (25%) and *Trichostrongylus* spp. (23%). A preliminary experiment was conducted to evaluate the lowest concentration giving 100% paralysis.

Ruta chalepensis methanol extract and its essential oil were tested on L3 GIN at a dose range of 0.02–0.20 and 0.40–6.30 mg/ml, respectively, and EC_{50} values were calculated. Pure compounds 2-nonanone, 2-undecanone and 2,4-decadienal, constituents of *R. chalepensis* extract, were tested individually at a concentration range of 0.03–1.60, 0.40–3.90 and 0.02–0.65 mg/ml, respectively. Moreover, (*E,E*)-2-4-decadienal, furfural, salicylaldehyde and epigallocatechin gallate were tested on L3 at dosages ranging from 0.30 to 2.30, 0.10 to 3.00, 0.35 to 6.00, and from 0.70 to 5.45 mg/ml, respectively. A stock solution of methanol extract was diluted with distilled water containing the polysorbate surfactant Tween 20, while the stock solution of essential oil was prepared in ethanol and successively diluted with water containing Tween 20. Stock solutions of test compounds were prepared using distilled water containing Tween 20 and, to

overcome insolubility, methanol was used. For the same reason, solutions of SAL and ECG were prepared using aqueous 0.1 M phosphate-buffered saline solution (PBS). Therefore, the solvents were used as carriers in treatment wells and as controls for death/paralysis correction in untreated wells. Final concentrations of solvent and Tween 20 in treatment wells never exceeded 1 and 0.3%, respectively. Aqueous solutions of PBS and methanol were used as negative controls, while levamisole was the positive control. Stock solutions of LEV were prepared at 1 mg/ml using water. Larval bioassays were performed in Cellstar 96-well cell-culture plates (Greiner Bio-One, Kremmunster, Austria) and each treatment consisted of 25 L3 per well (Martin & Le Jambre, 1979). The larval paralysis test was replicated six times per experiment and was carried out in the dark (Gill *et al.*, 1991) and at room temperature (Szewezuk *et al.*, 2006). L3 were analysed at 1, 24 and 48 h for pure compounds and 96 h for the extracts, with the aid of an inverted microscope (Euromex, The Netherlands) at 40× magnification. Nematodes were ranked into two categories: motile and immotile/paralysed. The classification of the larvae into immotile/paralysed was ascertained by absence of motility during an observation period of 6–8 s.

Data analysis

The percentages of L3 paralysed during the bioassay tests were corrected by elimination of the natural paralysis in the negative control according to the Schneider Orelli formula (Puntener, 1981):

$$\text{corrected \%} = \frac{\text{mortality \% in treatment} - \text{mortality \% in control}}{100 - \text{mortality \% in control}} \times 100.$$

The corrected percentages of treated L3 were analysed by Probit analysis for the determination of EC₅₀ values using Minitab 16 software (Minitab Inc., State College, Pennsylvania, USA) and a probability of 0.05 was used. EC₅₀ values indicate the concentration of the compound required for 50% paralysis after correction of percentages for natural paralysis. The results were expressed as EC₅₀ ± SD mg/ml. Moreover, linear regression was used to evaluate dose–response relationships.

Results

Mass spectrometry coupled to gas chromatography was used for the chemical characterization of the essential oil. According to GC–MS analysis, the essential oil comprised sabinene, α-pinene, limonene, 2-nonanone, 2-nonanol, geijerene isomer, geijerene, 2-decanone, octyl acetate, 2-undecanone, 2-dodecanone and 2-tridecanone (fig. 1). Mass chromatographic characteristics along with the percentage composition of the oil are reported in table 1. Levels of 2-nonanone and 2-undecanone were 25.3 and 24.0%, while limonene and octyl acetate were 12.8 and 10.4%, respectively. The oil yield was 1.09 ± 0.05% w/w expressed on a dry basis. Overall GC–MS analysis allowed the identification of 12 compounds, comprising

89.1% of the total oil composition; other unknown substances were present at low concentrations (table 1).

When REO and RME were tested for anthelmintic activity, a clear dose-dependent effect was observed. EC₅₀ values after 1 day of exposure in test solution to *R. chalepensis* methanol extract and its essential oil were 0.10 ± 0.08 and 1.29 ± 1.10 mg/ml, respectively. This result was confirmed 96 h after treatment, with an EC₅₀ of 0.10 ± 0.06 mg/ml for RME and 1.91 ± 1.61 mg/ml for REO. Furthermore, a linear relationship between the mean values of paralysis percentage and the log concentration of the tested solution was observed, with coefficient of determination (*R*²) values at 96 h of 0.981 and 0.984 for REO and RME, respectively. Levamisole was used as the positive control, with EC₅₀ values at 24 and 48 h after treatment of 0.63 ± 1.15 and 0.66 ± 0.65 mg/l, respectively (table 2). 2-Decanone, 2-nonanone and 2-undecanone showed anthelmintic activity with EC₅₀ values at 24 h after treatment of 0.07 ± 0.06, 0.25 ± 0.29 and 0.88 ± 0.73 mg/ml, respectively (table 2); these activities were confirmed at 48 h with EC₅₀ values of 0.11 ± 0.08, 0.25 ± 0.22 and 1.06 ± 1.05 mg/ml, respectively. A linear relationship between the mean values of paralysis percentage and the log concentration of solution tested was observed, with *R*² values at 48 h of 0.994 for UND, 0.978 for NON and 0.959 for DEC, respectively (table 2). For comparison, we tested the anthelmintic activity of four phytochemicals – 2,4-decadienal, furfural salicylaldehyde and epigallocatechin gallate. These compounds exhibited the highest activity at 24 h with EC₅₀ values of 1.03 ± 0.79, 1.83 ± 1.50, 2.12 ± 2.09 and 2.10 ± 2.14 mg/ml, respectively, while ECG at 48 h had an EC₅₀ value of 1.79 ± 1.72 mg/ml (table 2). The four compounds studied showed a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested, with *R*² values of 0.933, 0.901 and 0.970 at 24 h for DDI, FUR and SAL, and 0.887 and 0.847 at 48 h for ECG and LEV, respectively (table 2).

Discussion

According to other authors (Günaydin & Savci, 2005; Dob *et al.*, 2008; Ntalli *et al.*, 2011; Ali *et al.*, 2013; Haddouchi *et al.*, 2013; Caboni & Ntalli, 2014; Akkari *et al.*, 2015), 2-undecanone and 2-nonanone were the major constituents of the essential oil. Also Mejri *et al.* (2010) and Tounsi *et al.* (2011) reported 2-undecanone as the predominant compound, but followed by 2-decanone (Mejri *et al.*, 2010) or 2-nonanol (Tounsi *et al.*, 2011). The oil yield was 1.09 ± 0.05% w/w expressed on a dry basis. Ntalli *et al.* (2011) reported a yield of REO of 0.36 ± 0.10%, Tounsi *et al.* (2011) described yields in the range of 0.39–2.46% for leaves, flowers, stems and fruits, whereas Günaydin & Savci (2005) found a yield of 0.70%. Results are in accordance with the variability of different plant organs, the altitude, the climate, the soil where plants grew, the harvest period and the extraction technique used (Ntalli *et al.*, 2011; Tounsi *et al.*, 2011; Bouzidi *et al.*, 2012). Furthermore, the chemical profile of the methanol extract of *R. chalepensis* obtained by time of flight mass spectrometry coupled to liquid chromatography was in accordance with the profile of the extracts studied by

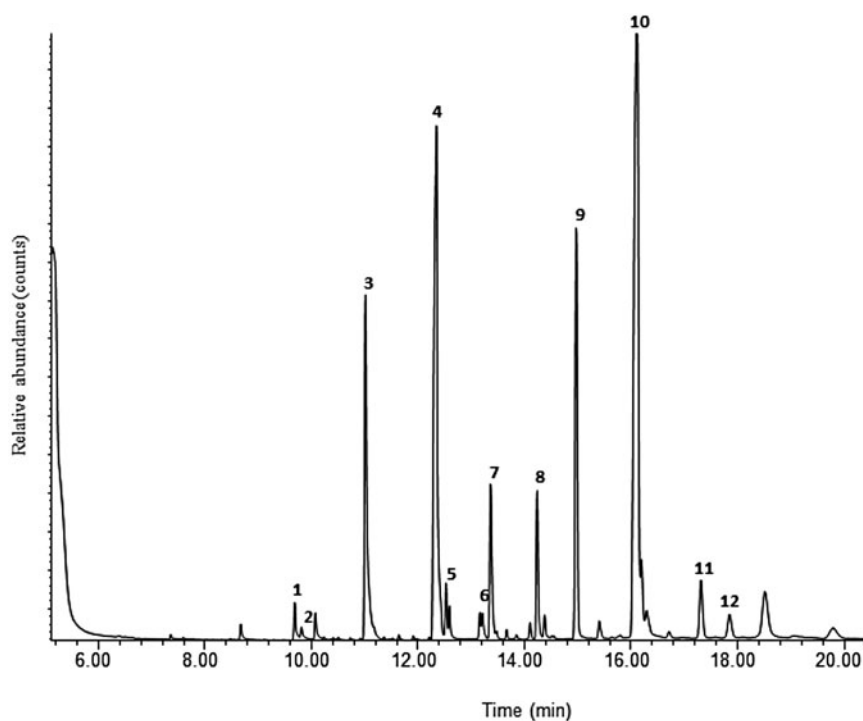


Fig. 1. GC–MS chromatogram of *R. chalepensis* L. essential oil to show peaks of: (1) sabinene, (2) α -pinene, (3) limonene, (4) 2-nonanone, (5) 2-nonanol, (6) geijerene isomer, (7) geijerene, (8) 2-decanone, (9) octyl acetate, (10) 2-undecanone, (11) 2-dodecanone and (12) 2-tridecanone. The remaining peaks are unknown.

Table 1. GC–MS analysis of *Ruta chalepensis* L. essential oil (REO). For each compound the molecular weight (MW) and two characteristic fragment ions with different mass to charge (m/z) ratios are shown.

Compound	Retention time (min)	Retention index	MW	Characteristic ions (m/z)	Percentage composition
Sabinene	9.70	976	136	93, 77	0.89
α -Pinene	9.82	980	136	93, 69	0.80
Limonene	11.02	1031	136	68, 67	12.82
2-Nonanone	12.32	1091	142	58, 71	25.31
2-Nonanol	12.49	1098	144	55, 69	1.28
Geijerene isomer	13.19	1137	162	79, 94	1.02
Geijerene	13.34	1137	162	79, 94	5.72
2-Decanone	14.17	1192	156	58, 71	3.65
Octyl acetate	14.86	1211	172	56, 70	10.42
2-Undecanone	15.91	1291	170	58, 71	24.01
2-Dodecanone	17.00	1369	184	58, 71	2.17
2-Tridecanone	17.45	1392	198	58, 71	1.05

Ntalli *et al.* (2011), where rutin, angelicin and 8-methoxy-psoralen were the main constituents, and by Günaydin & Savci (2005), where psoralen, chalepin and chalepensis were identified (data not shown). Nematode paralysis was evaluated by counting moving larvae with the use of an inverted microscope, as suggested by Gill *et al.* (1991). In accordance with Martin & Le Jambre (1979), larvae were considered immotile or paralysed when there were no observable movements during 6–8 s. Considering the high variability of percentage content of nematode species in real samples, *R. chalepensis* methanol

extract showed highest anthelmintic activity if compared with its essential oil. At 48 h *R. chalepensis* secondary metabolites 2-decanone, 2-nonanone and 2-undecanone showed an anthelmintic activity of 0.11 ± 0.08 , 0.25 ± 0.22 and 1.06 ± 1.05 mg/ml, respectively (table 2). A light micrograph of nematodes treated with DEC is shown in fig. 2. For comparison (*E,E*)-2,4-decadienal, an anthelmintic component of *Ailanthus altissima* (De Martino & De Feo, 2008), showed an EC_{50} of 1.09 ± 0.88 mg/ml. In addition, the *in vitro* anthelmintic activity of the secondary metabolites furfural, salicylaldehyde and epigallocatechin

Table 2. EC₅₀ (mg/ml) ± SD and coefficient of determination (R²) values of tested compounds on larval nematodes 1, 24 and 48 h after treatment.

Compound	Time of treatment					
	1 h		24 h		48 h	
	EC ₅₀	R ²	EC ₅₀	R ²	EC ₅₀	R ²
2-Decanone	0.26 ± 0.23	0.938	0.07 ± 0.06	0.891	0.11 ± 0.08	0.959
2-Nonanone	> 1.60	–	0.25 ± 0.29	0.915	0.25 ± 0.22	0.978
2-Undecanone	4.04 ± 3.33	0.998	0.88 ± 0.73	0.898	1.06 ± 1.05	0.994
2,4-Decadienal	1.23 ± 0.82	0.981	1.03 ± 0.79	0.933	1.09 ± 0.88	0.924
Furfural	2.60 ± 1.26	0.730	1.83 ± 1.50	0.901	2.18 ± 1.19	0.796
Salicylaldehyde	3.05 ± 1.97	0.944	2.12 ± 2.09	0.970	2.89 ± 2.50	0.922
Epigallocatechin gallate	2.59 ± 1.37	0.828	2.10 ± 2.14	0.866	1.79 ± 1.72	0.887
Gallic acid	No activity	–	No activity	–	No activity	–
Levamisole	0.0016 ± 0.0006	0.964	0.0006 ± 0.001	0.974	0.0007 ± 0.0006	0.847

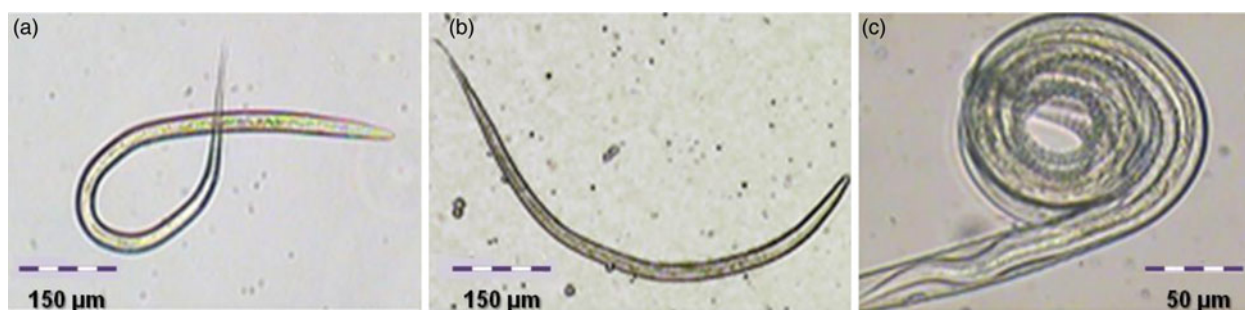


Fig. 2. Third-stage strongyloid larvae: (a) untreated; and treated with (b) 2-decanone at 0.20 mg/ml and (c) levamisole at 1.2 mg/l, both after 24 h.

gallate on sheep GIN was studied. FUR was also found to be a volatile nematicidal component of *A. altissima* (Ntalli *et al.*, 2010) while SAL was reported to be active against *Meloidogyne incognita* (Caboni *et al.*, 2013). These phytochemicals showed a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested (EC₅₀ values at 48 h of 2.18 ± 1.19 and 2.89 ± 2.50 mg/ml for FUR and SAL, respectively). On the other hand, when we tested ECG, we recorded an anthelmintic activity of 1.79 ± 1.72 mg/ml at 48 h. Molan *et al.* (2003) reported that ECG possesses ovicidal and larvicidal activity against *T. colubriformis* *in vitro*. Interestingly, gallic acid did not show anthelmintic activity at the concentrations tested (table 2). Moreover, levamisole anthelmintic activity (EC₅₀ value of 0.63 ± 1.15 mg/l at 24 h) can be compared with data reported by Martin & Le Jambre (1979), who obtained an LC₅₀ against *Ostertagia* spp., after 24 h of treatment, between 1.3 and 9.0 µg/ml. Lower EC₅₀ values can be explained hypothetically by the different species tested.

The present investigation clearly indicated that the *R. chalepensis* plant extracts RME and REO showed good anthelmintic activity against GIN if compared with other botanical extracts (Ademola *et al.*, 2004; Maciel *et al.*, 2006; Kamaraj *et al.* 2011, Elandalousi *et al.*, 2013), while they were less active if compared with the conventional anthelmintic, levamisole. Moreover, their constituents 2-undecanone, 2-nonanone, 2-decanone, together with

(*E,E*)-2,4-decadienal, were the most active tested compounds. A lower anthelmintic activity was recorded for furfural, salicylaldehyde and epigallocatechin gallate. Taking these results together, the use of phytochemicals is an opportunity for the development of valid alternatives for control of strongyle infestations in small ruminant herds. In conclusion, these compounds may find an application in the future as anthelmintics in veterinary practices. On the other hand, more studies are needed to evaluate the mode of action of these active compounds and to optimize the anthelmintic activity through studies of structure–activity relationships.

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Conflict of interest

None.

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