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Linalyl acetate against larvae of *Haemonchus* spp. and *Trichostrongylus* spp. that affects ruminants: considerations about the hormetic effect

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Research Paper

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Abstract

Active components from plants are an alternative therapy to parasite control, addressing the widespread multidrug resistance populations. Linalyl acetate (LA), an ester abundantly found in plants of the genus *Lavandula*, was tested in vitro against third-stage larvae (L3) of *Haemonchus* spp. and *Trichostrongylus* spp. using the larval migration test at 0.89, 2.24, 4.47, 8.95, 17.9, 35.8, 71.6, and 143.2 mg/ml. After an initial incubation of 18 h, the total content of each tube was transferred to a 24-well plate that allowed active L3 to migrate through a nylon mesh (second incubation). Although LA exhibited 100% efficacy in reducing larval migration at 8.95 and 17.9 mg/ml, it showed reduced activity (5%) at 143.2 mg/ml. The data revealed a hormetic biphasic response characterised by an inverted U-shaped concentration-response curve. While hormesis has been previously documented in insecticidal and allelopathic contexts, this study reports the occurrence of hormesis induced by a phytochemical component against two species of nematodes for the first time. This distinctive stimulation-and-inhibition effect should be considered when selecting new compounds for preclinical testing.

Introduction

Gastrointestinal parasites significantly cause infection and reduce welfare in small ruminants (Oliveira *et al.* 2017). In tropical and subtropical regions, *Haemonchus contortus* is the most prevalent nematode affecting ruminants, leading to apathy, lesions in the intestinal mucosa, and anemia (Zajac and Garza 2020). The second most frequent gastrointestinal nematode of sheep in Brazil is *Trichostrongylus colubriformis*, which causes weight loss, morbidity, and reduced food intake (Carvalho *et al.* 2021). Although several diagnostic methods are available, parasite control predominantly relies on the preventive use of anthelmintics (Molento *et al.* 2021). This strategy has led to the selection of multidrug resistance to *H. contortus* (Amaral *et al.* 2021), prompting research into new active compounds (Bortoluzzi *et al.* 2021; Selzer and Epe 2021; Soldera-Silva *et al.* 2018).

Several studies on humans and animals have tested active plant components to control parasites (Chaaban *et al.* 2019; Garbin *et al.* 2021). Esters are plant derivatives constituting the volatile base of essential oils (EO), terpenes, and other compounds. These compounds often have an intensely sweet and fruity odor, particularly during peak production when plants fully bloom (Elsharif *et al.* 2015). Linalyl acetate (LA) is an ester derived from linalool, extracted from *Lavandula angustifolia* EO through supercritical carbon dioxide extraction and from *L. officinalis* via steam distillation (Lis-Balchin *et al.* 1998; Altun and Yapici 2022). A substantial amount of LA is obtained from *L. angustifolia* (79.8%) and *L. officinalis* (34.9%) EO. Moreover, LA exhibits analgesic, anti-inflammatory, anxiolytic, antimicrobial, and sedative properties and is effective against burns and common skin lesions (Cavanagh and Wilkinson 2002). However, LA has yet to be tested against the nematodes *H. contortus* and *T. colubriformis*.

Hormesis is an adaptive stress response (Jentho *et al.* 2018) that is highly relevant to explaining biological organisation and risk assessment in toxicology, microbiology, medicine, and public health (Calabrese 2018). It refers to a biphasic pharmacological response where initial drug doses elicit a maximal effect. In contrast, higher doses result in a diminished/suppressive outcome, potentially reaching low or zero efficacy (Calabrese 2018). A hormesis curve for plant compounds has been documented; for instance, Papanastasiou *et al.* (2017) reported that limonene induced a typical hormetic insecticidal effect against the Mediterranean fruit fly *Ceratitis capitata*. This study aimed to determine the motility effect of LA against larvae of *Haemonchus* spp. and *Trichostrongylus* spp. and to report the hormetic effect of LA on these nematodes.

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Methodology

Plant material and nematode parasites

Pure LA was acquired from Quinari (Ponta Grossa, Brazil), reference 1331-1, produced by vapor pressure of 0.111 mm Hg at 25°C. The component was extracted from the flowers of *L. officinalis* Mont Blanc. The material was kept in a capped amber bottle until testing, when it was solubilised in distilled water containing 2% v/v Tween 80.

Fecal samples were collected from four naturally infected male White Dorper sheep from a farm located in Pinhais, Brazil (-25.38638, -49.12646). The material was subjected to a modified coprological technique (Gordon and Whitlock 1939) for quantifying nematode eggs, using 4 g of feces in 26 ml of saturated sucrose solution, and the result was multiplied by 25. Subsequently, fecal samples were mixed with an equal volume of vermiculite, moistened, and incubated at 25°C for 10 days to obtain third-stage larvae (L3). After that, the coproculture was then placed in a Baermann apparatus to collect L3. All larvae were identified at the genus level, according to van Wyk and Mayhew (2013).

The project was approved by the Animal Ethics Committee (CEUA) of the Agricultural Sciences Sector of the Federal University of Paraná, protocol 001/2019.

Larval migration inhibition test – LMT

The LMT was employed to determine the effect of LA on L3 following the methodology described by Demeler *et al.* (2010) with modifications. L3 were incubated in 2-ml opaque airtight microtubes. Approximately 200 L3 were incubated in the presence of LA at concentrations of 0.89, 2.24, 4.47, 8.95, 17.9, 35.8, 71.6, and 143.2 mg/ml. After the first incubation of 18 h, the total content of each tube was transferred to a 24-well plate where a 25 µm mesh separated two environments (upper and lower). The plate was sealed with parafilm to prevent volatilisation and incubated for another 24 h at 28°C under constant light. Subsequently, the mesh was carefully removed, and the L3 were counted under an inverted Optiphase INV-403 microscope (van Nuys, USA).

Statistical analysis

The percentage of L3 that did not migrate was determined for all controls and from each LA concentration. Each sample was tested in four replicates, including a negative control of distilled water (NC), a control using 2% Tween 80 (CT), and a positive control using 20% nitroxynil (C-NTX). A nonlinear regression analysis of the response to the concentrations was performed. Pharmacological comparison and efficacy were determined on a log scale (X = log X). A four-parameter logistic equation with variable slope was chosen using GraphPad Prism 6.1 (San Diego, USA). The efficacy was calculated according to the following formula:

$$Migration(\%) = (L3\,from\ control\ group - L3\,from\ treated\ group / L3\,from\ control\ group) \times 100$$

Results and discussion

L3 identification before testing revealed the presence of *Haemonchus* spp. (47.7%) and *Trichostrongylus* spp. (38.5%). As parasite populations were collected from the same farm with no previous drug resistance record, we assume a similar distribution after incubation. The LMT indicated that the three lowest concentrations of LA exhibited a concentration-dependent effect (stimulation) on the larvae with a linear correlation of 0.950 (Table 1). At doses of 8.95 and 17.9 mg/ml, the inhibition of L3 motility was 49% and 59% (plateau), respectively. At concentrations of 35.8, 71.6, and 143.2 mg/ml, the results showed a steady and proportional decrease in L3 migration inhibition of 44.7, 28.3, and 5.2%, respectively. The NC and CT did not affect L3 migration. NTX exhibited 91.5% efficacy (Table 1). According to Bosly (2022), in Saudi Arabia, the knockdown rate of lavender oil at 5%, containing 6.7% LA, was 95.5% in adults of *Culex pipiens*. In larvae, lavender oil induced an 87.2% mortality after 60 minutes of exposure (LC_{50} = 301.1 ppm). Mantovani *et al.* (2013) demonstrated 100% activity of 200 µg/ml of LA against adult forms of *Schistosoma mansoni* in Australia. The LC_{50} values against *S. mansoni* at 24 and 120 h incubation were 117.7 and 103.9 µg/ml, respectively.

Table 1. Percentage (%) of nematode larval inhibition of migration and standard deviation (SD) after treatment with linalyl acetate (LA)

LA concentration (mg/mL)	% Inhibition (+/- SD)
0.89	2.63 (± 1.73)
2.24	34.45 (± 3.05)
4.47	36.29 (± 4.16)
8.95	45.75 (± 3.59)
17.9	52.59 (± 4.18)
35.8	44.74 (± 0.87)
71.6	28.26 (± 5.50)
143.2	5.19 (± 2.67)
CT-Tween 80	2.32 ± (0.88)
CH-water	0.1 (± 0)
C-NTX	91.54 (± 2.07)

Figure 1 illustrates the hormetic effect of LA, demonstrating an inverted U-shaped curve characterised by an increase, plateau, and subsequent decrease in efficacy. The data suggest that at higher concentrations, LA promotes an opposing mechanism of action, blocking the parasite's receptors to the compound and preventing future interactions (Calabrese and Mattson 2017). The hormetic mechanism may be based on the premise that

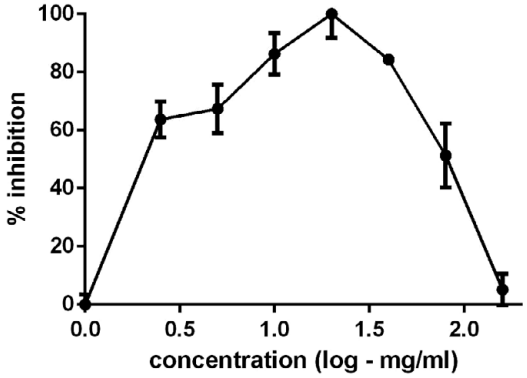

Figure 1. Hormetic concentration-dependent effect of linalyl acetate against *Haemonchus* spp. and *Trichostrongylus* spp.

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low stress activates or positively induces responses to the molecules. The hormetic effect may also depend on the organism's nutritional status, temperature, and other preconditions (Calabrese 2018).

It is well known that the biological effect of EO depends on the presence or absence of various components (Mande and Sekar 2020). The hormetic effect of EO has been described in *Drosophila melanogaster* (fruit fly), *Musca domestica* (housefly), and *Acheta domesticus* (cricket) (Cutler *et al.* 2022). The EO of *Rosmarinus officinalis* (rosemary), which contains 27.5% of α-pinene in fully ripe fruits, stimulated the growth of radish roots and aerial parts at doses up to 300 µl/L but decreased the stimulus at doses greater than 1200 µl/L (Alipour and Saharkhiz 2016). When used against the adult stage of *Ceratitis capitata* (Benakeio fly), limonene increased the fertility and life expectancy of the species in low concentrations, but these effects diminished considerably at higher doses (Papanastasiou *et al.* 2017). *Citrus myrtifolia* (Chinotto orange) exhibited a hormetic impact on *Lolium multifolium* (Italian ryegrass) seeds, containing 76.8% limonene and 1.0% LA (Caputo *et al.* 2020). The hormetic effect was also observed in A375 cells during tests with an aqueous emulsion of bergamot (*Citrus bergamia*) and orange (*C. sinensis*) EO containing 43.3% limonene (Alexa *et al.* 2022). *Thymus vulgaris* (thyme), which contains thymol as the main component (33%), also showed a hormetic effect on UMSCC1 human cancer cells with a decrease in its lethal effect at concentrations above 0.54 mg/ml (Sertel *et al.* 2011).

A crucial issue also relates to the ecological relevance of hormesis in an incorrect drug resistance diagnostic. Drug resistance is an evolutionary phenotypic response represented by a lack of parasite reduction (i.e., *H. contortus*) (Silva *et al.* 2022) manifested by parasite paralysis and death. Under a hormetic effect, this lack of reduction may not demonstrate resistance, as organisms might moderately adapt their behavior or even increase their fitness in response to changing environments (Costantini 2019; Jalal *et al.* 2021). Kishimoto *et al.* (2017) reported the transmission of a resilient phenotype for oxidative stress and proteotoxicity through hormetic mechanisms in *Caenorhabditis elegans*. The hormetic effect was transmitted epigenetically upon exposure to various stressors during developmental stages, providing survival advantages for generations after controlled stress exposure. Therefore, the inverted U-shaped efficacy of a phytochemical component against two species of ruminant parasites observed in vitro should be taken with caution, as reports of lack of effectiveness are a consequence of two major stress factors of frequent drug use, and the combination of products (Molento and Brandão 2020). This distinctive stimulation-and-inhibition effect should also be considered when selecting new compounds for preclinical testing.

Conclusion

LA was effective against free-living stages of *Haemonchus* spp. and *Trichostrongylus* spp. However, the compound exhibited hormetic activity at doses above 35.8 mg/ml, demonstrating a reduction in efficacy to only 5% at 143.2 mg/ml. This study is the first to report the hormetic effect of a phytochemical component against nematode parasites of ruminants.

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Competing interest. The authors declare no conflict of interest.

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