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Phytochemical modulation of P-Glycoprotein and its gene expression in an ivermectin-resistant *Haemonchus contortus* isolate in vitro

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ABSTRACT

Haemonchus contortus is the most important gastrointestinal nematode in small ruminant systems worldwide and has developed resistance to several drugs, including ivermectin (IVM). IVM is not only a veterinary drug but also a safe, broad-spectrum, antiparasitic drug used in humans. One of the main IVM-resistance mechanisms in *H. contortus* involves P-glycoprotein (PgP), a trans-membrane transport protein that rids worm cells from toxic molecules. This study aimed to evaluate the anthelmintic activity of IVM, alone or combined with main terpenes of essential oils (alpha-terpinene, beta-citronellol, beta-pinene, citronellal, limonene, menthol, and terpinolene) and with phenolic compounds (epicatechin, epigallocatechin, gallocatechin, pentagalloylglucose, procyanidin, and quercetin). All compounds were tested, alone or combined with IVM, against susceptible (*Hc*S) and resistant (*Hc*R) isolates of *H. contortus* through the larval development test (LDT) and the adult motility assay (AMT) using verapamil (VP), a known PgP modulator, as a control. Results for the LDT determined that the lethal concentration required to kill 50% of nematodes (LC_{50}) with IVM was 10 times greater (0.01 μg/mL) for *Hc*R than for *Hc*S (0.001 μg/mL). The combination IVM + VP inhibited the activity of PgP in *Hc*R resulting in a LC_{50} = 0.002 ug.mL^{-1}. Although limonene was the least effective and alpha-terpinene the most effective terpene when tested alone against *Hc*R, the best combinations were IVM + limonene and IVM + quercetin both produced LC_{50} = 0.002 μg/mL (similar to IVM+VP) which were chosen for subsequent tests. Because adult parasites are the final target for anthelmintics, IVM was evaluated in *Hc*S (LC_{50} = 0.067 μg/mL) and *Hc*R (LC_{50} =164.94 μg/mL) through the AMT. Results obtained with IVM + VP (LC_{50} = 0.020 μg/mL) in *Hc*R were similar to IVM + limonene (LC_{50} = 0.028 μg/mL) and outperformed IVM + quercetin (LC_{50} = 1.39 μg/mL). RNA extracts from *Hc*R adult worms exposed to IVM, IVM+VP, and IVM + limonene were evaluated for PgP expression by RT-PCR. For most concentrations, PgP-9 was significantly more expressed in worms treated with IVM alone than in worms treated with IVM + VP or IVM + limonene. Our results suggest that limonene is involved in the modulation of the PgP-9 gene and that it can restore the activity of IVM in the *Hc*R isolate down to levels seen in *Hc*S. Limonene is one of the main compounds found in citrus peel and has the potential to be both safe and affordable if used in combination with IVM to restore its anthelmintic effects against multi-drug-resistant *H. contortus* isolates. Our results also suggest that we may be more successful by combining natural products with failing commercial anthelmintics than trying to find natural substitutes for them.

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1. Introduction

Avermectins are a class of 16-membered macrolide lactones produced by the bacteria *Streptomyces avermitilis*, an actinomycete isolated from a soil sample from Japan 42 years ago an that synthesizes a complex of eight different avermectins (Burg et al., 1979). The researchers Satoshi Omura and William Campbell shared the 2015 Nobel Prize in Medicine for their respective discoveries on avermectin anthelmintic effects and in the treatment of infections caused by roundworms (https://www.nobelprize.org/prizes/medicine/2015/press-release/). However, several reports of worldwide *Haemonchus contortus* resistance to avermectins have been published. In sheep, ivermectin resistance in *H. contortus* was first reported in 1987, approximately ten years after it was launched by the veterinary pharmaceutical industry (Carmichael et al., 1987).

Some hypotheses have been considered in studies about macrocyclic lactone resistance in nematodes, such as the involvement of permeability (P) glycoprotein (gP) or Glycoprotein-P, which is a cell-membrane hydrophobic protein with high molecular weight, high lipophilicity, and a member of the ATP-dependent transporters. PgPs are responsible for cell efflux of xenobiotics by preventing their intracellular accumulation and protecting the cells from potentially toxic molecules (Ferreira et al., 2014).

Overexpression of genes encoding for PgP is believed to be the main cause of multidrug resistance (MDR) (Varma et al., 2003). MDR is determined not only by the expression of these genes, but also their level of expression, which is crucial for cells to receive signaling and to be able to perform changes in the system that will lead to anthelmintic resistance (Kerboeuf, et al., 2003).

The presence of PgP was verified in all life stages of *H. contortus*, from eggs to adult parasites (Xu et al., 1998; Kerboeuf et al., 2003). PgP was also found in the cuticle of first- and third-stage larvae. Hence, the presence of PgP in these structures constitutes a protective biochemical mechanism during all the development stages of the parasite (Riou et al., 2005).

The modulation of PgP can increase the availability of drugs inside the cell by competing with the drug binding site or by blocking the ATP hydrolysis reaction, increasing drug efficacy (Azeredo et al., 2009). The modulation of ATP-dependent transporters associated with MDR, such as PgP, may lead to the reversal of MDR, the main mechanism associated with the loss of effect of macrocyclic lactones (David et al., 2018).

PgP modulators are classified into 4 generations according to toxicity scores and include synthetic drugs and compounds of natural origin. Phytochemicals are natural compounds produced by plants and are included in the fourth generation, which contains drugs of low mammalian toxicity (Bastos et al., 2015). Some phytochemicals such as terpenes and phenolic compounds can induce or inhibit drug metabolism.

Citronellal, beta-citronellol, alpha-terpinene, terpinolene, beta-pinene, and menthol, which were evaluated in this study, were classified as potential PgP modulators in studies with human cells (Yoshida et al., 2006). Phenolic compounds such as catechins, pentagalloyl glucose, and procyanidins were also evaluated (Kitagawa et al., 2004) (Table 1). Procyanidins are members of the proanthocyanidin (or condensed tannins) class of flavonoids. They are oligomeric compounds, formed from catechin and epicatechin molecules. The monoterpene limonene has been evaluated for its PgP modulation effect in bacteria (Table 1). The co-administration of phytochemical modulators of PgP with ivermectin (IVM) may improve the anthelmintic efficacy of IVM and other anthelmintic drugs.

The interaction of modulators that can decrease MDR needs to be thoroughly studied. Several PgP modulators are associated with macrocyclic lactones in different species, indicating their potential use to increase the effectiveness of drug treatments against different parasites (Lifschitz et al., 2010; Dupuy et al., 2003; Ballent et al., 2007; Alvinerie et al., 2008).

Resistant *H. contortus* isolates had their genome fully sequenced and may show high expression of different PgP genes with at least 10 genes related to ABC transporters being observed (Laing et al., 2013). Two genes were identified as the main ones involved in resistance: PgP-2 (expressed at the pharynx, first portion of the intestine, and adjacent nervous tissues, suggesting the protection of nematodes from the effects of macrocyclic lactones) (Reyes-Guerrero et al., 2020; Godoy et al., 2015) and PgP-9 was overexpressed in the female reproductive system (uterus of adult *H. contortus*) of drug- resistant nematodes (Godoy et al., 2016).

The aims of this work were to evaluate the effect of phytochemicals alone and in combinations with ivermectin in both sensitive and resistant isolates of *H. contortus* through the larval development test (LDT) and the adult motility test (AMT); then to compare gene expression in a resistant isolate and verify if there was any interference of PgP modulators with the PgP-9 gene in worms treated with IVM and IVM combined with VP.

2. Material and methods

Drugs and phytochemicals evaluated: ivermectin (Sigma Aldrich ®), Verapamil (ChemCruz ™), alpha-terpinene and menthol (AlfaAesar®), beta-pinene and limonene (Sigma Aldrich ®), citronellal and citronellol (Dierberger®), epicatechin, epigallocatechin, gallocatechin, and quercetin (Cayman Chemical®), pentagalloyl glucose and procyanidin (ChemCruz®). The drugs and phytochemical compounds used were

Table 1
Monoterpenoid and phenolic compounds that have been previously evaluated for their activity as P-glycoprotein (*PgP*) modulators and reversal of multi-drug resistance (MDR) in bacteria and cancer cells.

	Pub Chem ID	Compound	Molecular Weight g/mol	Activity in *PgP*	References
Terpenoids	6321424	Ivermectin	875.1	–	–
	62969	Verapamil	491.1	*PgP* inhibitor	Huber et al. (2010)
	7462	Alpha-terpinene	136.23	Inhibit *PgP*-mediated transport in cancer cells	
	14896	Beta-pinene	136.23		Yoshida et al. (2006)
	7794	Citronellal	154.25		Yoshida et al. (2006)
	81263	Citronellol	156.26	Increase cell accumulation of the *PgP* substrate	Yoshida et al. (2006)
	1254	Menthol	156.26		Eid et al. (2015)
	440917	Limonene	136.23	Reversal resistance in bacterial MDR	Araujo et al. (2021)
	11463	Terpinolene	136.23	Impaired the growth of cancer cells with high *PgP* expression	Calcabrini et al. (2004)
Phenolics	72276	Epicatechin	290.27	Inhibits *PgP* in mouse lymphoma cells	Martins et al. (2010)
	65064	Epigallocatechin	458.4	Cell accumulation of *PgP* substrates	Kitagawa et al. (2004)
	9882981	Gallocatechin	306.27	Drug resistance reversal in KB-A-1 cells	Kitagawa et al. (2004)
	65238	Pentagaloylglucose	940.7	Inhibits *PgP* in MDR KB-C2 cells	Kitagawa et al. (2007)
	11250133	Procyanidin	578.5	Inhibits the function and expression of P-gP	Zhao et al. (2013)
	5280343	Quercetin	302.23	Inhibits *PgP* in MDR cancer cells	Mohana et al. (2016)

molecular standards with at least 98% purity and were acquired from commercial sources. Previously published studies on the evaluated compounds are shown in Table 1.

2.1. Haemonchus contortus isolates

- *Hc*S: susceptible isolate of *Haemonchus contortus,* named as Rs*Hc*o1, and maintained at the Universidade Estadual Paulista (UNESP), Botucatu campus, State of São Paulo, Brazil (Amarante et al., 2017).

- *Hc*R: multidrug resistant isolate of *H. contortus* (Almeida et al., 2010), named as Sp*Hc*o2, and maintained at UNESP, Botucatu campus, State of São Paulo, Brazil (Amarante et al., 2017).

In anthelmintic efficacy evaluations based on worm counts after necropsy, the SpHco2 resistant isolate presented high level of resistance to albendazole, levamisole, ivermectin and closantel (Almeida et al., 2010), while the RsHco1 susceptible isolate displayed high susceptibility to albendazole, levamisole and ivermectin (Echevarria et al., 1991).

Four lambs were treated with monepantel (Zolvix ™ Novartis) at 2.5 mg/ kg of live weight (LW) and levamisole phosphate 18.8% (Ripercol® L 150F- Zoetis) at 1 mL/20 kg LW to eliminate nematodes acquired through natural infections. After the parasite-free *status* was confirmed through 3 replicates of fecal egg count (FEC) according to Ueno and Gonçalves (1988), artificial infections were performed with approximately 4000 *H. contortus* larvae in their L3 stage. Two lambs were infected with the susceptible *Hc*S isolate and two lambs with the resistant *Hc*R isolate.

Animal procedures and management protocols were approved by the Ethics Committee to the Animal Welfare (CEUA-IZ) in Nova Odessa, São Paulo, Brazil, and received the protocol number 257–18.

2.2. Larval development test (LDT)

The LDT was performed following the methodology of Hubert and Kerboeuf (1992) and Bizimenyera et al. (2006). The egg recovery procedure was performed according to Coles et al. (1992).

The chemicals IVM, verapamil (VP), and all phytochemical compounds were solubilized in 0.5% DMSO and evaluated individually to obtain average lethal concentrations to kill 50% (LC_{50}) and 90% (LC_{90}) of the evaluated population of each *H. contortus* isolate (Table 3). VP was used in this study as a positive control because it represents a class of PgP inhibitors (Huber et al., 2010). The compounds and concentrations tested are listed in Table 2.

Treatment combinations and doses were determined from the LC_{90} values of each compound (Table 3) where the LC_{90} of IVM was added to LC_{90} of Verapamil and also to each LC_{90} of phytochemicals to create each combination.

All tested compounds were diluted tenfold to achieve target decreased concentrations with the minimal concentration being one that allowed larvae to reach their full development. Serial dilutions of the drugs and phytochemicals were made to be applied on 100 L1 larvae with six replicates for each concentration. After 6 days of incubation at 27 C and 80% relative humidity, the L1 larvae were evaluated under an inverted microscope to make a differential count between larvae that reached the L3 developmental stage and the ones that did not.

Data were analyzed by SAS Probit statistical software to determine LC_{50} and LC_{90} of each compound or their combinations as independent variables (doses) after logarithmic transformation (log dose). Significant differences between ($P \leq 0.05$) means of the concentrations were determined by Tukey's studentized range test.

Table 2
Concentrations of phytochemicals and ivermectin evaluated in LDT.

Compound	Concentration (ug/mL)
Ivermectin	1000; 10; 1; 0.1; 0.01; 0.001; 0.0001; 0.00001
Verapamil	1000; 100; 10; 1; 0.1
Alpha-terpinene/Beta citronellol Beta-pinene/Citronellal	1000; 100; 10; 1; 0.1; 0.01; 0.005
Limonene/Menthol/Terpinolene	3000; 300; 30; 3; 0.3; 0.03; 0.003
Epicatechin/Epigallocatechin Gallocatechin/Quercetin	100; 10; 1; 0.1; 0.01; 0.001; 0.0001; 0.00001

2.3. Adult Motility test (AMT)

The AMT was performed following the methodology of Hounzangbe-Adote et al. (2005) with adaptations of O'Grady and Kotze (2004) to evaluate the effects of the best result of associations obtained in LDT.

Adult females *H. contortus* were obtained from the abomasum of infected lambs with two isolates immediately after euthanasia. The parasites were washed, kept in saline solution (37 C), and placed in 24-well plates containing culture medium (RPMI-1640, Hepes buffer, glucose, bovine serum, antibiotics, and fungicide). IVM, VP, limonene, quercetin, and respective combinations with IVM (compounds that showed the most promising results in LDT) were solubilized with DMSO (0.5%) and diluted in the culture medium.

The concentration of each compound was tested in both *H. contortus* isolates, on a decreasing decimal scale. The dosages were established according to the results obtained with the LDT. IVM was evaluated at the first concentration of 10^3 µg/mL; VP 2×10^3 µg/mL; Quercetin 4×10^3 µg/mL and Limonene 10^3 µg/mL. The concentration was always determined based on IVM and decreased tenfold as follows: 10^3 until 10^{-5}. In *Hc*S the tests started in 10^1 decreasing to 10^{-5} µg/mL. The concentrations were tested in 6 replicates with 5 worms/replicate. Worm motility was verified by observation under an inverted microscope after 24 h of incubation at 37 C.

Data were analyzed by SAS Probit statistical software to determine CL_{50} and CL_{90} of each compound or compound combinations as independent variables (doses) after logarithmic transformation (log dose). Significant differences ($P \leq 0.05$) between means of the concentrations were determined by Tukey's studentized range test.

2.4. Analysis of PgP-9 expression in live adult females of HcR (resistant) H. contortus

The expression of the PgP-9 gene was performed with the best results observed for the AMT assay (IVM + terpenes) with surviving worms (*Hc*R) exposed to drugs at 10^1 to 10^{-5} g/mL. PgP-9 was chosen because of several studies that show a strong interaction between this gene and ivermectin (Godoy et al., 2016).

The RNA extracts were obtained from the samples of different treatments and concentrations tested with the AMT assay. Live worms were frozen in liquid nitrogen to preserve their RNA. RNA samples were extracted from a pool of 10 adult females of *H. contortus* with three biological replicates ($n = 30$) for each concentration of each treatment. The treatments IVM, IVM + VP, and IVM + limonene were evaluated at 10^1 to 10^{-5} µg/mL. The RNA extractions were performed with TRI Reagent (Sigma-Aldrich, St. Louis, USA, cat. N. T9424). For phase separation (protein, DNA, and RNA), 1-bromo-3-chloropropane, isopropanol (2-propanol), and 75% ethanol were used to form an RNA pellet. The concentration and purity of RNA samples were determined using a BioDrop UV/VIS spectrophotometer (Biochrom®, www.analitica.com.br) and the samples were also subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized on the DocTM XR + Gel System (Bio-Rad Laboratories Equipment, Hercules, CA, USA). All samples were adjusted to the final concentration of 125 ng/µL. cDNA synthesis was performed with RNA previously treated with DNase in a reaction using the High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (Ref. No. 4374967, Applied Biosystems, CA, USA).

The expression of the PgP-9 gene for each concentration/treatment was analyzed by quantitative RT-PCR on a Rotor Gene Q (QIAGEN, Hilden, Germany) with HOT FIREPol® Evagreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia) following the manufacturer's recommendation, with each sample evaluated in duplicate. The data obtained by the

Table 3
Lethal concentrations (LC_{50} and LC_{90} μg/mL) obtained in larval development test with anthelmintic susceptible (*Hc*S) and resistant (*Hc*R) strain of *Haemonchus contortus* in response to compounds evaluated isolated and respective fiducial limits (FL).

Drugs and phytochemicals	LC_{50} *Hc*S	FL	LC_{90} *Hc*S	FL	LC_{50} *Hc*R	FL	LC_{90} *Hc*R	FL
Ivermectin	0.001	0.0008–0.0015	0.01	0.007–0.013	0.01	0.005–0.022	0.4	0.218–0.994
Verapamil	4.10	1.52 6.70	43	29.7–59.6	7.26	5.68–8.75	30	25.3–39.1
Citronellal	0.33	0.10–0.66	8.4	5.10–16.2	3.67	1.11–9.07	41.9	12.0–407
Limonene	242.01	181.66–314.29	1568	1105–2511	256.33	189.65–354.99	1701	1061–3380
Menthol	48.28	18.72–82.43	579	386.6–1060	0.03	0.005–0.068	0.36	0.22–0.65
Terpinolene	26.72	15.68–38.57	1211	179.4 – 6278.7	188.48	153.25–233.57	598	444.8–923.2
Alpha Terpinene	13.49	7.04–21.13	142	88.3–290.9	2.87	0.677–5.63	30	16.5–99.6
Beta Citronellol	0.49	0.23–0.89	20	9.6–57.8	39.98	34.85 – 45.97	202	125.8–489.9
Beta Pinene	9.12	3.14 – 22.26	134	33.3–1076	12.44	4.33 – 19.96	271	31.9–1850.9
Epicatechin	1.28	0.57–2.06	6.6	3.78–24.3	0.34	0.24–0.45	1.17	0.868–1.784
Epigalocatechin	5.24	2.77–11.84	29	12.83–275.2	12.54	9.64–16.50	45	31.2–84.2
Galocatechin	3.78	2.39–4.97	10	7.65–16.3	1.30	0.35–2.36	8	4.51–26.5
Pentagall	60.11	50.06–81.81	128	91.1–284.2	41.45	32.82–53.44	117	84.3–206.0
Procianidin	10.38	8.41–12.80	27	21.2–42.0	9.38	7.38–11.96	24	17.7–38.5
Quercetin	0.41	0.28–0.45	110	0.26–225	0.005	0.002–0.009	0.08	0.045–0.223

reverse transcription technique, followed by the quantitative polymerase chain reaction in real-time (RT-qPCR) were analyzed by a mixed linear model with software SAS "Mixed"(SAS Institute, Cary, NC) to evaluate the levels of expression of the P glycoprotein gene triggered by different interactions. The dose effects were tested within each treatment, and for the comparison between treatments, each dose was analyzed separately. The comparisons of the mean relative changes in gene expression per treatment group (IVM, IVM+VP, and IVM + limonene), by concentration (10^{1}–10^{-5} μg/mL), were performed using the Mann-Whitney test, with the level of significance set at $p < 0.05$.

3. Results

The LC_{50} values for IVM, VP, and each phytochemical were obtained from the larval development test (LDT) of strains of *H. contortus* sensitive (*Hc*S) and resistant (*Hc*R) to anthelmintics, and the results are shown in Table 3.

The larval response of both isolates to limonene, menthol, procyanidin, and pentagalloyl glucose was similar, indicating a pattern in the response mechanisms for the respective isolates. The categorization in 'susceptible' or 'resistant' isolates was based on tests with commercial anthelmintics (Almeida et al., 2010; Amarante et al., 2017). The anthelmintic responsiveness of phytochemicals may be different for each isolate.

When tested alone, the most effective monoterpene in *Hc*S was citronellal, which presented a LC_{50} of 0.33 μg/mL. In the resistant isolate, the most effective was alpha-terpinene, which presented a LC_{50} of 2.87 μg/mL. In both isolates, the least effective monoterpene was limonene, which presented a LC_{50} in *Hc*S: 242.01 μg/mL and *Hc*R: 256.33 μg/mL.

Quercetin was the most effective phenolic providing a LC_{50} of 0.41 μg/mL in *Hc*S and 0.005 ug/mL in *Hc*R. The least effective phytochemical was pentagalloyl glucose (*Hc*S: 160.33 μg/mL and *Hc*R: 41.45 μg/mL). Procyanidin had similar values of anthelmintic activity in both isolates (*Hc*R: 9.38 μg/mL and *Hc*S: 10.38 μg/mL).

When tested in *Hc*R, IVM had an LC_{50} of 0.01 μg/mL while in susceptible *Hc*S it had a LC_{50} of 0.001 μg/mL. When combined with VP, IVM had an increased effectiveness in *Hc*R (LC_{50}: 0.002 μg/mL), confirming the expected PgP inhibitory effect of VP. However, there was no increased effect of IVM+VP in the *Hc*S isolate, as observed for the LC_{50} of IVM+VP compared to IVM alone.

The combination IVM+quercetin resulted in the lowest value for LC_{50} (*Hc*S: 0.002 μg/mL and *Hc*R: 0.002 μg/mL) with an efficacy similar to the one observed for the control IVM + VP (0.002 μg/mL). Gallocatechin combined with IVM showed a similar value (0.01 μg/mL) when compared to IVM tested alone. When IVM was combined with limonene and beta-pinene its efficacy increased in the *Hc*S isolate. However, only the IVM + limonene combination was able to increase IVM efficacy in the *Hc*R isolate (Table 4).

For the screening with adult worms (the final target of our tested compounds), IVM, VP, limonene, quercetin, and their respective combinations, were evaluated in the Adult Motility Test (AMT) for anthelmintic-resistant (*Hc*R) and anthelmintic-susceptible (*Hc*S) isolates of *H. contortus* females with the results shown in Table 5.

In AMT, *Hc*S had a LC_{50} of 0.067 μg/mL for IVM while the LC_{50} in *Hc*R was 164.94 μg/mL. When VP was combined with IVM, it was observed a significant increase in efficacy in *Hc*R (0.17 μg/mL). The effect of IVM is partially expressed by the paralysis of muscular structures that cause worm death and this could explain the wide range between the upper and lower limits.

VP alone presented 0.056 μg/mL in *Hc*S and 0.34 μg/mL in *Hc*R. In both isolates, quercetin was not effective, and it was not possible to estimate its LC_{50}. However, when IVM + quercetin were combined, the increase in IVM efficacy was as good as for the combination IVM + VP, with a LC_{50} of 0.295 μg/mL in *Hc*S and 1.39 μg/mL in *Hc*R (Table 5). Limonene + IVM was the best effective combination in both *Hc*S and *Hc*R with a LC_{50} of 0.028 μg/mL in *Hc*R.

For the detection of genetic interactions between phytochemicals and IVM observed in AMT, the expression of PgP was performed in the same worms exposed to phytochemicals and VPM in the AMT assay (Fig. 1). Limonene was chosen as it had the best result in combination

Table 4
Lethal concentrations (LC_{50} μg/mL) obtained in the larval development test with anthelmintic susceptible (*Hc*S) and resistant (*Hc*R) isolate of *Haemonchus contortus* in response to several combinations with ivermectin (IVM).

IVM combination with phytochemicals	LC_{50} *Hc*S	Fiducial limits 95%	LC_{50} *Hc*R	Fiducial limits 95%
IVM + Verapamil	0.001	0.0004–0.002	0.002	0.001–0.003
IVM + Citronellal	0.08	0.01–13.14	0.02	0.012–0.018
IVM + Limonene	0.00016	0.00001–0.0007	0.002	0.0009–0.004
IVM + Menthol	0.008	0.0002–0.010	0.04	0.026–0.060
IVM + Terpinolene	6.55	0.2–10.6	0.030	0.021–0.043
IVM + Alpha-Terpinene	0.02	0.004–0.06	0.02	0.009–0.042
IVM + Beta-Citronellol	0.008	0.0004–0.01	0.03	0.020–0.036
IVM + Beta-Pinene	0.0002	0.00008–0.0005	0.08	0.073–0.095
IVM + Epicatechin	0.05	0.006–3.232	0.034	0.031–0.037
IVM + Epigalocatechin	0.01	0.0009–0.01	0.03	0.034–0.045
IVM + Galocatechin	0.01	0.0006–0.01	0.013	0.011–0.014
IVM + Pentagalloyl.	0.002	0.00116–0.00609	0.02	0.012–0.025
IVM + Procianidin	0.01	0.003–0.25	0.02	0.016–0.022
IVM + Quercetin	0.00002	0.00002–0.00005	0.002	0.001–0.004

Table 5
Lethal concentrations (LC_{50} μg/mL) obtained through the motility test with *Haemonchus contortus* adult females resistant (*Hc*R) and susceptible (*Hc*S) to ivermectin (IVM) and other anthelmintic drugs.

Drugs and phytochemicals	LC_{50} *Hc*S	Fiducial limits 95%	LC_{50} *Hc*R	Fiducial limits 95%
IVM	0.067	0.016–0.295	164.94	70.71–399.63
VP	0.056	0.037–0.086	0.34	0.07 – 6.10
IVM+VP	0.050	0.025–0.110	0.17	0.07 – 0.35
Limonene	1.063	0.670–1.736	7.36	4.95–11.10
IVM+Limonene	0.048	0.029–0.081	0.028	0.017–0.043
Quercetin	[a]	[a]		
IVM+Quercetin	0.295	0.169 0.599	1.39	0.444–4.720

[a] at the highest dose tested was not effective (*Hc*S: 4 mg/mL and *Hc*R: 40 mg/mL).

with IVM. When comparing the genetic expressions, PgP-9 was significantly ($p < 0.05$) more expressed (or upregulated) at the concentrations 10^{1} μg/mL, 10^{0} μg/mL, 10^{-2} μg/mL, 10^{-3} μg/mL and 10^{-5} μg.mL^{-1} in the treatment of IVM when compared to IVM + VP and IVM + limonene (Fig. 1). No differences were observed between IVM+VP and IVM+limonene at any concentration.

4. Discussion

In the present study, the effectiveness of phytochemicals was evaluated for their use alone or in combination with IVM. Results were compared to IVM alone and combined with VPM through the larval development test (LDT) and the adult motility test (AMT) in female worms of IVM-susceptible (*Hc*S) and IVM-resistant (*Hc*R) isolates. The PgP-9 gene expression of *Hc*R worms exposed to IVM alone and in combinations with phytochemicals were also evaluated through RT-PCR.

An increase in the efficacy of IVM was observed for the combination IVM+VP in LDT and AMT in the *Hc*R isolate. A few previous studies reported similar results and attributed this increased efficacy to the potential of VP to modulate PgP activity in *H. contortus* in vitro tests (Molento and Prichard, 1999; Lespine et al., 2007; Bartley et al., 2009). In susceptible isolates, no effect was observed with the CL_{50} for IVM

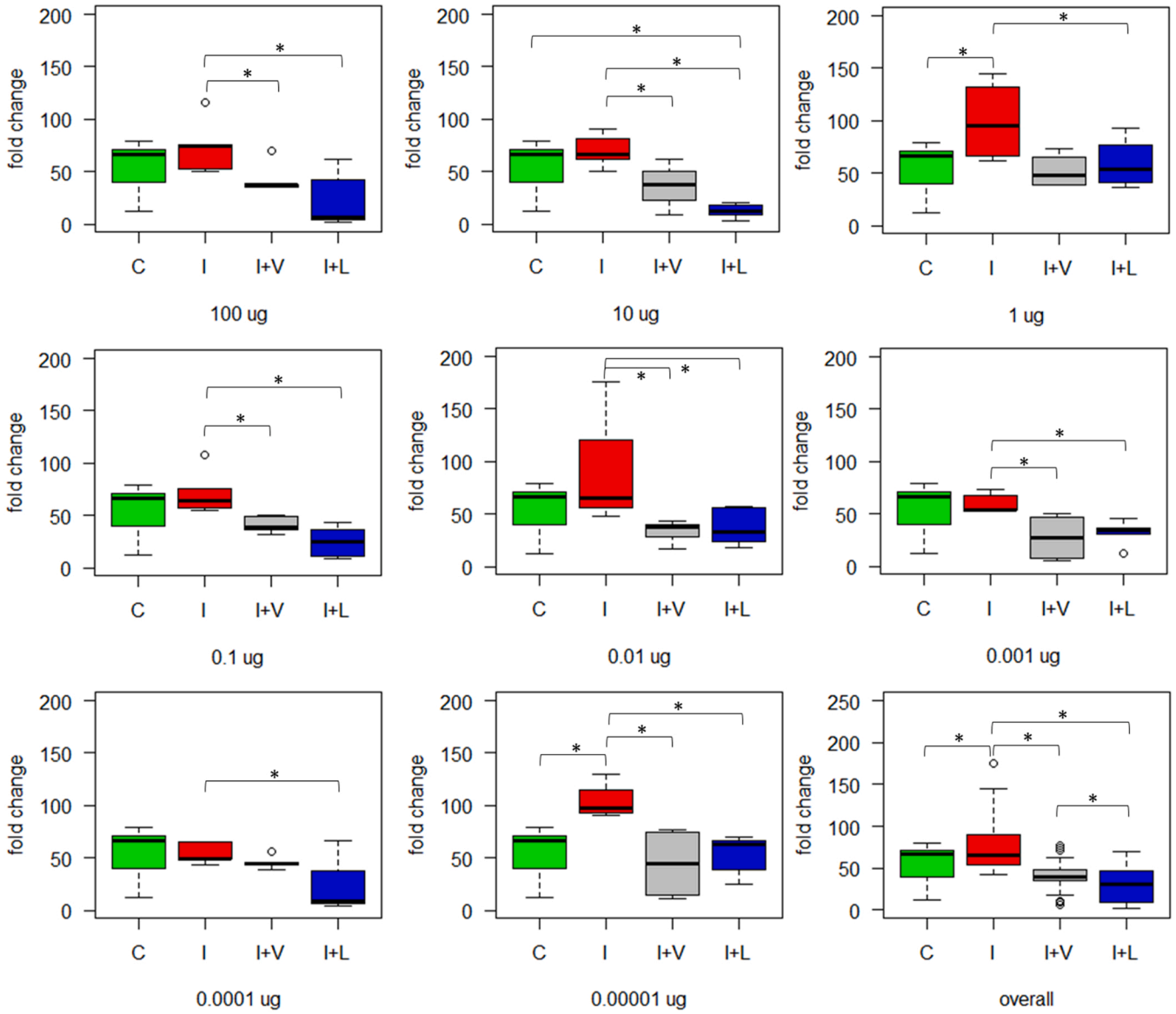

Fig. 1. Level of relative gene expression of PgP-9 analyzed in live *Haemonchu contortus* resistant isolate treated with Ivermectin (red), Ivermectin + Verapamil (gray) and Ivermectin + Limonene (blue) between the concentrations of 10^{1} to 10^{-5} μg measure in RNA samples collected from adult females and Control (green). Comparisons between the different concentrations were made for each treatment. * = significantly different at $P < 0.05$.

(0.001 μg.mL^{-1}) being similar to IVM + VP (0.001 μg.mL^{-1}), supposedly because there was no mechanism associated with PgP that could be influenced by VP.

Several mechanisms are related to anthelmintic resistance to macrocyclic lactones in *H. contortus*, such as the one involving beta-tubulin (Baltrusis et al., 2020) or the enzymes related to cytochrome P450 (Kellerová et al., 2019). However, the overexpression of ABC transporters, such as P-glycoproteins, is the most cited mechanism due to its intrinsic involvement with ivermectin resistance (Mate et al., 2022), thus being the target of our study.

A mechanism of IVM resistance in *H. contortus* highly studied is the PgP, a transmembrane protein that is a product of the MDR gene and acts in the efflux of drugs and xenobiotics from the nematode cell (Lanusse et al., 2018). PgP activity can be modulated using VP, an anti-arrhythmic drug that blocks calcium channels and is widely used to revert resistance to anti-tumor therapy (although VP is very toxic and causes many side effects in vertebrates). Several studies have shown the potential of VP to modulate the activity of PgP from *H. contortus* and reverse IVM resistance (Borges et al., 2011; Molento and Prichard, 2001).

The phytochemicals evaluated in this study have low toxicity as they are present in many fruits, vegetables, and spices. They were previously investigated for the potential to modulate PgP or reverse the multidrug resistance: Epicatechin, epigallocatechin, gallocatechin, pentagalloyl glucose (Kitagawa et al., 2004), quercetin (Heckler et al., 2014; Mohana et al., 2016; Borges et al., 2020), epigallocatechin (Mohana et al., 2018), procyanidin (Zhao et al., 2013), alpha-terpinene, beta-citronellol, beta-pinene and citronellal (Yoshida et al., 2006), limonene (Zhang and Lim, 2008), menthol (Eid et al., 2015), and terpinolene (Calcabrini et al., 2004) were previously presented as PgP modulators. Each one was tested alone and in combination with IVM to determine potential synergistic or antagonist effects.

Not all combinations of phytochemicals with ivermectin improved the outcome like IVM+VP. When used alone, limonene had low activity in LDT with both isolates. However, in combination with IVM, it was among the most effective anthelmintic combinations in both isolates. In AMT the results were similar. Limonene was able to increase the efficacy of IVM in *Hc*S and in *Hc*R being considered the most promising of the phytochemicals evaluated. The potential of limonene to interfere in drugs metabolism was the subject of a study with other drugs, such as midazolam. Limonene interfered in the hydroxylation of midazolam decreasing its metabolic degradation (Zhang and Lim, 2008) and have been reported to modulate the function of human nicotinic acetylcholine receptors, (Lozon et al., 2016).

A recent in vitro study about the effect of phytochemicals associated with levamisole on *Oesophagostomum dentatum* demonstrated that limonene oxide potently inhibited a nicotinic receptor of acetylcholine (Choudhary et al., 2019). These authors also showed that limonene oxide and carvacrol produced a non-competitive inhibition of the acetylcholine receptor indicating that the activity of these compounds does not involve binding to the agonist-binding site to produce its effects. This demonstrates that limonene may have different ways to interact with receptors and create anthelmintic effects that are not triggered only by PgP.

Among phenolic compounds, quercetin presented the best efficacy in both isolates in LDT. The anthelmintic activity of quercetin has been reported previously by Klongsiriwet et al. (2015) in LDT with *H. contortus*. In AMT it was not possible to estimate the efficacy of quercetin due to the lack of anthelmintic efficacy at the highest dose tested. However, the combination of quercetin with IVM was able to increase the efficacy of IVM in *Hc*R and *Hc*S in LDT and AMT, confirming similar results of Borges et al. (2020). Heckler et al. (2014) also observed an increased efficacy of IVM + quercetin in resistant *Haemonchus placei*.

The combination IVM + quercetin in the susceptible *Hc* isolate presented a lower LC50 than in the resistant isolate. Although the categorization of susceptible or resistant isolates is given only based on commercial anthelmintics (Almeida et al., 2010; Amarante et al., 2017), the interaction of this phenolic compound in resistant *Hc* isolate needs to be investigated to elucidate the peculiarities with different degrees of resistance. Our results were also similar to the ones reported by Borges et al. (2020).

Differences found in the LDT or AMT, which assess the worm at different stages of development, suggest that *PgP*'s expression in developing larvae may have a greater affinity for quercetin than in adults or no affinity at all for quercetin (Borges et al., 2020). Furthermore, the levels of PgP expression can vary according to the helminthic stage of development (Dicker et al., 2011; Sarai et al., 2013; Raza et al., 2015). Additional defense mechanisms such as enzyme systems and transporters may occur in adults and with greater intensity in resistant isolates (Bartley et al., 2012). Similar differences observed for *Haemonchus* treated with the same drug/secondary metabolite (phytochemical) combination at different life stages were also observed by other authors. Lespine et al. (2012) explained that there may be a specificity in the efflux mechanisms that can interfere with the efficacy of IVM in some stages of nematode development.

However, the lack of activity of compounds such as epicatechin, as seen here, may be related to its chemical structure as studies have shown that methylation would be a reasonable modification to improve its activity (Lee et al., 2005). The compounds epigallocatechin, epicatechin, and gallocatechin, although widely used as PgP modulators due to their low cytotoxicity in some literature reports (Sano et al., 2001; Schauss et al., 2006) did not increase IVM efficacy in this case. Studies suggest that the action of compounds such as those mentioned above occurs synergistically with each other; so, it would be feasible to assume that their combined use in animals would produce better bioactivity than when used separately.

Although there are some studies involving the anthelmintic activity of phytochemicals (Katiki et al., 2017; Katiki et al., 2011) against *H. contortus* in vitro and some in vivo (Squires et al., 2010; Silva et al., 2021), we did not find any studies involving the exact mechanism of action of these phytochemicals in *H. contortus*. However, compounds such as limonene have been reported to modulate the function of human nicotinic acetylcholine receptors (Lozon et al., 2016).

A fact to be highlighted in this study is that the terpenes combined with IVM followed the same pattern observed for phenolic compounds in both isolates. In combination with IVM, all terpenes increased anthelmintic efficacy, supposedly due to the additive or synergistic effect caused by their combination with anthelmintic drugs (Yoshida et al., 2006; Zhou et al., 2007). However, these are not the only mechanisms that explain this increase in efficacy. The activity of PgP modulators occurs through mechanisms related to the expression of the PgP gene or by competitive inhibition with membrane proteins or by phosphorylation of transport proteins. Studies about the potential for reversion of resistance by some phytochemicals and reversal of resistance to multiple drugs in tumor cells have shown that the activity of the modulators is dependent on the dose and substrate combinations (Wink et al., 2012; Eid et al., 2015).

The lipophilic properties of terpenoids are responsible for their affinity for the cell phospholipid bilayer (Dorman and Deans, 2000). The target of terpenes is the membrane, and this is probably the reason they interfere with the organism's susceptibility to drugs (Nazzaro and Fratianni, 2013) as in cases where the drug tolerance is related to efflux pumps located inside the cell membrane, such as PgPs.

The anthelmintic activity of IVM on *H. contortus* is often evaluated through LDT and AMT (Malik et al., 2020), although there are few reports about the anthelmintic activity of purified phytochemicals combined with IVM. Therefore, these results could increase our understanding of phytochemicals for future studies. Some of the phytochemicals evaluated in this study demonstrated the potential to increase the efficacy of IVM in LDT and AMT. Although it is a well-known fact that structural changes present in different nematode life stages of *H. contortus* may interfere with the anthelmintic drug uptake process,

such as diffusion through cuticles in larval stages and intestinal cells in the adult stage (Lifschitz et al., 2017), some studies suggest that PgP is present and active in all life stages of *Haemonchus* (Riou et al., 2005).

Regarding the PgP gene expression study, the combinations IVM + VP and IVM + limonene resulted in lower gene expression in the resistant *H. contortus* isolate, compared to the treatment IVM alone in all concentrations evaluated. The mode of action by which these drugs can revert multidrug resistance has not been fully established leading to further questions on how phytochemicals may have modes of action similar to VP on PgP, such as by suppressing the expression of resistance genes. Other phytochemicals were studied for their effect on genetic expression (Nabekura et al., 2005) and the ATP hydrolysis and substrate ABC transporters are tightly coupled by most compounds.

Gilleard and Beech (2007) demonstrated that the resistance to ivermectin can be increased with the continued use of this anthelmintic and one study reported a change in allelic frequency with the gradual increase of ivermectin, which works as a genetic selective pressure. Yusa and Tsuro (1989) showed that, in cancer cells, VP binds directly to PgP, suggesting that it reverses resistance by competitively inhibiting the site used for drug transport. Studies have shown that VP significantly increased the anthelmintic effect of IVM and moxidectin against selected isolates of *H. contortus* in guinea pigs and gerbils (Xu et al., 1998, Molento and Prichard, 1999).

The genome of *H. contortus* has been entirely sequenced and at least 10 genes among ABC transporters have been described (Laing et al., 2013). Several studies reported changes in expression levels of different PgPs genes that may be associated with parasitic resistance in *H. contortus* (Blackhall et al., 2008; Sarai et al., 2013; 2014; Alvarez et al., 2015; Godoy et al., 2015, 2016; Raza et al., 2015; David et al., 2018; Mate et al., 2022). Previously to this study, several tests with different PgPs (1, 2, 3, 4, 9, 10, 11, 12, and 16) were performed. Among all *PgP*s evaluated, only *PgP-9* was significantly ($P < 0.05$) more expressed in the resistant strain (data not shown). Comparing the genetic expressions of each concentration and between treatments, *PgP-9* was significantly upregulated ($P < 0.05$) in worms treated with IVM alone when compared to IVM + VP, as previously corroborated by Prichard and Roulet (2007) and Lloberas et al. (2013).

It is possible to select resistant worms induced by IVM used as a monotherapy. This resistance is associated with higher levels of PgP mRNA than non-selected isolates (Xu et al., 1998), which leads us to suggest that overexpression of PgP in this parasite may be a consequence of the selective pressure caused by IVM.

Our results with limonene, the main component of *Citrus* peel essential oil, and its similar role to VP in vitro, suggest that an in vivo study should be conducted to validate that the blockage of PgP could potentially increase the clinical efficacy of IVM in animals infected with resistant strains of *H. contortus*.

Some phytochemical modulators used in this study show anthelmintic potential, as observed in the results of the tests with susceptible and drug-resistant isolates, and should be tested in vivo. The combination with anthelmintic molecules such as ivermectin can be beneficial because each compound can act by different mechanisms, acting at different sites of the target parasite that acquired drug resistance (Lanusse et al., 2018). The investigation of in vivo kinetics of phytochemicals tested individually or combined with IVM, and the increased efficacy of IVM when combined with limonene in this study, may lead to the improved efficacy of IVM against resistant *H. contortus*.

5. Conclusion

In our study, both in vitro tests conducted in IVM-resistant *H. contortus*, determined that the combination of IVM + limonene improved IVM tolerance in a drug-resistant *H. contortus* strain similar to the control combination of IVM + VP, although no effect was observed when limonene was used alone. The benefits of finding natural products that may synergize or revert IVM tolerance in pathogenic parasites are of enormous benefit to the health of humans and animals worldwide. Our results also suggest that we could make faster advances in gastrointestinal parasite control in livestock by pairing natural compounds with failing commercial anthelmintics.

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CRediT authorship contribution statement

P.A. Pacheco: Investigation. **H. Louvandini:** Methodology, Validation. **B.C.R. Wedy:** Investigation. **J.C. Ribeiro:** Investigation. **R. Giglioti:** Formal analysis. **C.J. Verissimo:** Writing – original draft. **J.F. S. Ferreira:** Conceptualization, Writing – review & editing. **A.F.T. Amarante:** Methodology, Validation. **L.M. Katiki:** Conceptualization, Methodology, Project administration, Funding acquisition.

Conflict of interest

The authors declare that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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