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Combination of quercetin and ivermectin: *In vitro* and *in vivo* effects against *Haemonchus contortus*

Dyego Gonçalves Lino Borges[a,*], Marcelo Augusto de Araújo[a], Carlos Alexandre Carollo[b], Aline Regina Hellmann Carollo[c], Adrian Lifschitz[d], Mário Henrique Conde[a], Mariana Green de Freitas[a], Zelina dos Santos Freire[a], Juliane Francielle Tutija[a], Matheus Takemi Muchon Nakatani[a], Fernando de Almeida Borges[a]

[a] Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Filinto Müller 2443, Ipiranga, Caixa Postal 549, CEP: 79074-460, Campo Grande, MS, Brazil
[b] Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, Brazil
[c] Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição, Universidade Federal de Mato Grosso do Sul, Brazil
[d] Laboratório de Farmacologia, Centro de Investigação Veterinária de Tandil, (CIVETAN), (UNCPBA- CICPBA-CONICET), Faculdade de Ciências Veterinárias, Universidade Nacional do Centro, Tandil, Argentina

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ABSTRACT

The aim of the present study was to evaluate the *in vitro* effect of quercetin combined with ivermectin (IVM) on *Haemonchus contortus* larvae and adults with different resistance profiles and demonstrate the *in vivo* anthelmintic action of this combination when used in sheep naturally infected. The effect of combination was evaluated based on the analysis of the mean effective concentration (EC50) obtained for larvae using the larval migration inhibition test and for adults using the motility test on females. The tests with larvae and adults were conducted using isolates with different degrees of susceptibility to IVM (sensitive, intermediate and highly resistant). The *in vivo* effect was evaluated based on the reduction in the egg count (FEC) and reduction in the count of adult helminths recovered after parasitological necropsy. Using the combination of quercetin with IVM, it was observed that in larvae, quercetin did not significantly reduce the EC50 for IVM in the sensitive and highly resistant isolates, but led to a significant reduction in the EC50 for IVM in the intermediate isolate. In adults, quercetin did not significantly reduce the EC50 for IVM in any of the isolates. No significant effect of the combination was found regarding the reduction in FEC or total count of parasites. The results of the *in vitro* and *in vivo* tests performed in the present study on quercetin activity underscore the importance of evaluating resistance-reversing agents among different stages of parasite development as well as among isolates with different resistance profiles. The action of quercetin combined with IVM on the motility of *H. contortus* larvae and adults was influenced by the degree of resistance and development stage of the parasite. The combination was effective only on intermediate resistant larvae. No action of the combination against adults was found. Moreover, this combination, when administered through the intra-abomasal route, was not effective at reducing the FEC and parasite load of naturally infected sheep.

1. Introduction

Haemonchus contortus (Nematoda: Trichostrongylidae) is one of the most important parasites for sheep farming worldwide. It is a parasite with a direct life cycle, without migrations by the host organism and with a feeding habit of hematophagy that inhabits sheep abomasum, being particularly pathogenic for lambs (Muchiut et al., 2019) recently calved and lactating ewes (Armour, 1980). It usually causes hypoproteinemia, anemia, and anorexia in infected animals, which results in reduced productivity and, in many cases, mortality (Kaplan et al., 2004). The economic damage caused by the parasite is underestimated in many regions, such as Brazil. On the other hand, in Australia, losses of around $ 436 billion (AUD) per year are estimated (Lane et al., 2015). One factor that aggravates this economic damage is the anthelmintic resistance, which in some Southern countries, for example, has reached levels that make sheep farming unsustainable

* Corresponding author.
E-mail address: dyego.borges@ufms.br (D.G.L. Borges).

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(Jackson and Coop, 2000).

In this global scenario of anthelmintic resistance, the search for new strategies to control worms is urgent. One possibility of maintaining the use of drugs already available is by combining resistance modulators, drugs used to block or reduce the biochemical mechanisms of parasite resistance, so that antiparasitic efficacy can be restored (Lespine et al., 2008).

Quercetin is a flavonoid found in different *plant species,* many of which are used as food for humans and feed for farm animals (Besle et al., 2010). Quercetin has numerous biological activities (Rice-Evans, 2001; Havsteen, 2002; Spencer et al., 2004), including the ability to achieve the biochemical reversal of resistance to ivermectin (IVM) in larvae of the genus *Haemonchus* (Bartley et al., 2009; Heckler et al., 2014). This resistance-reversing effect has been attributed to the modulation of ATP-dependent transporters associated with multiple drug resistance, such as P-glycoprotein (P-gp), which is the main mechanism associated with nematode resistance to macrocyclic lactones (David et al., 2018).

In vivo evaluations of quercetin as an anthelmintic resistance-reversing agent are scarce in ruminants, despite the promising results demonstrated using *in vitro* methods (Bartley et al., 2009; Heckler et al., 2014). One *in vivo* study investigated the combination of quercetin and moxidectin (subcutaneous administration) in sheep (Dupuy et al., 2002). However, the authors did not evaluate the *in vivo* anthelmintic effect of the combination, but rather the influence of quercetin on the pharmacokinetic aspects of moxidectin, demonstrating an increase in its bioavailability. Therefore, there is no *in vivo* proof of the resistance-reversing effect of quercetin.

Drugs that are considered effective when evaluated using *in vitro* methods do not necessarily have proven *in vivo* effectiveness (O'Grady and Kotze, 2004). Indeed, the results of anthelmintic *in vitro* and *in vivo* evaluations are incompatible in some cases. For instance, ketoconazol is a P-gp modulator with corroborated *in vitro* activity against isolates of IVM-resistant helminths, and when evaluated *in vivo* and combined with IVM, an increase in the plasma concentration of IVM was found, but this did not lead to an increase in the effectiveness of the anthelmintic (Bartley et al., 2012). The reason for this incompatibility in the results may reside in the drug-host and drug-parasite interactions (Ballent et al., 2006).

Regarding the drug-host interaction, bioavailability and half-life of the drug exert an influence on the action of medications. For orally administrated quercetin, bioavailability is only 0.1% due to the extensive ruminal fermentation plus the fact that the metabolism and elimination of quercetin is extremely rapid (Berger et al., 2012; Gohlke et al., 2013). Other factors that have been suggested, but not yet proven, as having an influence on the effect of drugs include the life stage and degree of susceptibility of the parasite, which are aspects of the drug-parasite interaction. Free living stages are biochemically and physiologically different from parasitic stages with regard to many important aspects of the metabolism potential of drugs (see review by O'Grady and Kotze, 2004). Moreover, biochemical differences may also be associated with the degree of susceptibility of parasites, such that the resistance factor may represent a source of variation in the effect of anthelmintic and modulating drugs (Raza et al., 2015). Thus, tests in different stages of life, especially adult stages, which are one of the main targets of drugs with anthelmintic action, and tests involving helminths with different degrees of susceptibility are important to the screening of substances with action against nematodes (see review by O'Grady and Kotze, 2004), even before conducting *in vivo* tests, which constitute a subsequent step required of the drug evaluation process.

An assessment protocol for any anthelmintic substance or formulation should necessarily be based on the evaluation of candidate helminth drugs at different stages of development and susceptibility profiles and, if there is evidence of anthelmintic activity, *in vitro* tests should be performed to confirm the observed *in vitro* effect.

Therefore, the aim of the present study was to evaluate the *in vitro* effect of quercetin combined, for this first time, with ivermectin on *H. contortus* larvae and adults with different degrees of resistance and verify the anthelmintic action of this combination in an *in vivo* experiment involving its administration to sheep naturally infected by *H. contortus* ivermectin resistant.

2. Material and methods

An *in vitro* evaluation was performed of the anthelmintic action of quercetin combined with IVM on third-stage larvae and adults of *H. contortus* with different degrees of resistance. For such, the mean effective concentration (EC50) of IVM alone and in combination with quercetin was determined for larvae and adults. In the *in vivo* experiment, the anthelmintic action of the same combination was evaluated after administration through the intra-abomasal route in sheep naturally infected with an IVM-resistant isolate of *H. contortus*. This study received approval from the Ethics Committee on Animal Use of the Federal University of Mato Grosso do Sul (certificate number: 475/2012) and was conducted in accordance with all legally established ethical principles.

2.1. In vitro evaluation

2.1.1. Parasites

The following three isolates of *H. contortus* were used:

(i) RsHco1: isolate maintained at the *Universidade Estadual Paulista Professor Júlio de Mesquita Filho*, Botucatu campus, State of São Paulo, Brazil, kindly donated by Dr. Alessandro F. T. do Amarante (Amarante et al., 2017). This isolate is sensitive to IVM (EC50: 5.96×10^{-6}).
(ii) FAMEZHco1: field isolate maintained at Federal University of Mato Grosso do Sul in the city of Campo Grande, State of Mato Grosso do Sul, Brazil. This isolate has been exposed to different anthelmintics for more than eight years and is resistant to IVM (EC50: 1.87×10^{-5}).
(iii) ROHco1: field isolate acquired from a property in the State of Rondônia, Brazil. This isolate is considered highly resistant to IVM (EC50: 1.453×10^{-4}).

2.1.2. Drugs

Technical grade ivermectin (Sigma-Aldrich) and quercetin (Xi'na Frankherb Biotech Co. Ltd.) were used. For each drug, a stock solution was prepared at 10^{-2} M in dimethyl sulfoxide (DMSO). The solutions were divided into aliquots, frozen and stored at −20 °C until the tests.

2.1.3. Drug concentrations

IVM was used as concentrations of 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M and 10^{-9} M for RsHco1 as well as 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M and 10^{-7} M for both FAMEZHco1 and ROHco1. All concentrations were obtained based on Demeler et al. (2010), except saline solution (0.15 mol/l) was used rather than distilled water. Quercetin was used at a concentration of 10^{-5} M for all isolates (Heckler et al., 2014).

The same concentrations of IVM and quercetin were used in the tests with larvae and adults. The concentrations of IVM used in the tests for the determination of the EC50 of IVM were also maintained in the tests for the determination of the EC50 of the quercetin + IVM combination.

The concentration of DMSO was standardized at 0.5% in each well in all treatments. A negative control of 0.5% DMSO alone was included in each test. In the test with the combined drug, an additional control group was included: quercetin at a concentration of 10^{-5} M.

2.1.4. Larval migration inhibition test

2.1.4.1. Obtainment of larvae. For the maintenance of the isolates, six sheep were stabled in duly prepared brick stalls with a cement floor,

Table 1
Motility classification system for *H. contortus* females exposed or not-exposed to ivermectin or ivermectin + quercetin combination.
Source: Adapted from Kotze et al. (2012).

Motility score	Description
1	No movement
2	Limited movement (<75% of body: movement of one or both extremities of body, with central region of coiled or distended, but immobile). Body coiled, independently of movement. Minimal movement, movements with less intensity only at extremities of body, <10% movement.
3	Rapid sinusoidal movement involving entire body, >90% of body in motion. Significant movement: spread throughout more than 75% of body.

where they remained throughout the entire experiment. The animals received corn silage as feed (2.5% of live weight on a dry matter basis), appropriate mineral salt and water *ad libitum*. After the adaptation period, the animals were de-wormed with albendazole (10 mg/kg, oral administration, Ricobendazole, Ourofino Saúde Animal) combined with levamisole (10 mg/kg, oral administration, Ripercol, Zoetis) for five consecutive days, after which the animals were submitted to an egg count in the feces [fecal egg count (FEC)] using the McMaster technique (Gordon and Whitlock, 1939) with 1:25 sensitivity. As no parasite forms were found in the feces, the animals were monitored for an additional 15 days. With the negative FEC results during this period, the animals were considered to be free of helminth infection. The animals were then divided into three groups ($n = 2$). Each group was inoculated with one of the three *H. contortus* isolates (10,000 third-stage larvae per animal). Thirty days after inoculation, fecal samples were collected daily to determine the production of third-stage larvae.

Fecal cultures were performed following the methods described by Robert and Sullivan (1950). The cultures were incubated for seven days at 27 °C, followed by the extraction of third-stage larvae (L3s). The larvae were transferred to 45-ml Falcon tubes and stored in a refrigerator at 5–8 °C for an additional seven days until the tests.

2.1.4.2. Larval migration inhibition test. The ability of IVM either alone or combined with quercetin to inhibit the migration of *H. contortus* larvae was determined using the larval migration inhibition test described by Demeler et al. (2010), with modifications. Two tests were performed for each isolate: one for the determination of the EC50 for IVM and one for the determination of the EC50 for quercetin + IVM.

The L3s were submitted to the Baermann method using a sieve with a 25-μm mesh to ensure that only viable larvae were used. Viable larvae were collected from the Baermann apparatus and quantified to standardize the larval suspension as 100 L3s in 20 μL of saline solution. Using 24-well plates, 1690 μL of saline solution at 0.15 mol/l, 20 μL of the larval suspension and 90 μL of the drug solution (IVM alone or 45 μL of IVM and 45 μL of quercetin) were added to each well (six wells or replicates per treatment) for the determination of the EC50. Solutions twofold more concentrated were used for the determination of the EC50 of the combined drug to ensure that the desired concentrations would be reached when adding the smaller volume of the solution to each well. The final volume was 1800 μL per well. The larvae were then incubated, transferred to migration plates and quantified using the method described by Demeler et al. (2010).

2.1.5. Motility test with H. contortus females

2.1.5.1. Retrieval of adults. Four previously inoculated lambs (two inoculated with RsHco1, one with FAMEZHco1 and one with ROHco1) with FEC ≥ 4000 were euthanized. The abdominal cavity was opened and ligatures were placed in the transition between the omasum and abomasum as well as in the initial portion of the duodenum. The abomasum was removed immediately, placed in an appropriate thermal chest to avoid the cooling of the organ and sent to the laboratory. The organ was sectioned along the major curvature and immersed in saline solution (NaCl at 0.15 mol/l) heated to 37 °C. The females retrieved were placed in Petri dishes, washed, kept in saline solution and stored in a chamber at 37 °C until the motility tests.

2.1.5.2. Motility test. The ability of IVM alone or combined with quercetin to inhibit the motility of *H. contortus* females was determined using the motility test described by Demeler et al. (2014), with modifications. Two tests were performed for each isolate: one for the determination of the EC50 for IVM and one for the determination of the EC50 for quercetin + IVM.

Using 24-well plates, 1710 μL of saline solution at 0.15 mol/l, three *H. contortus* females and 90 μL of the drug solution [IVM alone (Test E) or 45 μL of IVM and 45 μL of quercetin (Test A4)] were added to each well (six wells or replicates per treatment) for the determination of the EC50. Solutions twofold more concentrated were used for the determination of the EC50 of the combined drug (Test A4) to ensure that the desired concentrations would be reached when adding the smaller volume of the solution to each well. The final volume was 1800 μL per well.

The females were then incubated with the different treatments for a total of eight hours. Motility was evaluated under a stereomicroscope after four, six and eight hours. At each evaluation time, motility was observed for one uninterrupted minute in each well and was classified on three levels: 1 = absence of motility; 2 = slow movements; and 3 = fast movements (Table 1).

2.2. In vivo evaluation

2.2.1. Coproparasitological evaluations and parasitological necropsy

2.2.1.1. Animals and experimental area. Eighteen male sheep aged five to eight months and naturally infected by a mixed isolate (95% *H. contortus* and 5% *Cooperia* spp.) were selected based on FEC counts among animals in a commercial herd in the region of Campo Grande, state of Mato Grosso do Sul, Brazil. The isolate was resistant to ivermectin, as demonstrated during a previous evaluation, when the reduction in the FEC was less than 80%. The selected animals (FEC > 300) were transferred to isolation stalls at the School of Veterinary Medicine and Animal Husbandry of the *Universidade Federal de Mato Grosso do Sul*, where they were kept during a 10-day adaptation period and throughout the entire experimental period (nine days), receiving corn silage (2.5% of live weight on a dry matter basis per day) and water *ad libitum*.

The coproparasitological methods (FEC and fecal cultures) were performed on three occasions prior to treatment (D-2, D-1 and D0). Based on the mean FEC on D0, the animals were divided in groups using an entirely randomized block design.

The groups were randomly assigned to the different treatments (quercetin, ivermectin and ivermectin + quercetin). The animals in Group I received 0.008 mg/kg of ivermectin; the animals in Group II received 0.008 mg/kg of ivermectin and 120 mg/kg of quercetin; and the animals in Group III (Group control) received 120 mg/kg of quercetin. Group III (quercetin) was used as a control group, since quercetin does not have nematodicide effect (Heckler et al., 2014). The quercetin group was also important to evaluate the possible effects of the drugs and administration protocols on the parasite population in the different treatments. The treatments were administered directly into the lumen of the abomasum through a puncture of the organ and access was obtained through a surgical route. The dose adopted for IVM was based on the luminal concentration of IVM in the abomasal fluid that is achieved

after the oral administration of the drug (Lloberas et al., 2012). The choice of dosage of quercetin took into account previously performed tests, which demonstrated that concentrations below 120 mg/kg did not potentiate the effect of ivermectin (unpublished data), the stability of quercetin in the formulation and the volume of the formulation to be administered. Dosages above 120 mg/kg would require much larger volumes of solution, which could hinder intra-abomasal administration of the treatment.

2.2.1.2. Drugs used. Quercetin: 6% quercetin (Xi'na Frankherb Biotech Co., Ltd.) was diluted in oleic acid (Sigma-Aldrich) and linoleic acid (Sigma-Aldrich) at a proportion of 6:4, giving rise to a suspension. The quercetin in oil suspension was used to increase the duration of the drug combination in the abomasum and the slow the release of quercetin into the abomasal fluid.

Ivermectin: commercial oral solution fo Ivomec – Merial Saúde Animal (batch: EA905/15).

2.2.1.3. Intra-abomasal administration of drugs. The animals were submitted to dietary fasting for 18 h with no water for six hours and subsequently weighed on a previously calibrated mobile scale (Coimma, ICM – 300). The animals were sedated with an intramuscular injection of xylazine (Calmiun, Agener União) 0.1 mg/kg. When the sedation was evident, the anterior half of the right abdominal wall was shaven. After local antisepsis, lidocaine (Lidovet, Bravet) 10 ml/animal was administered through subcutaneous and intramuscular injection on the incision line (straight line 10 cm in length beginning 5 cm posterior to the xiphoid cartilage and 3 cm above the origin of the umbilical fold). The animals were maintained individually in left lateral decubitus.

The rectus abdominis muscle was separated, followed by opening of the peritoneum, enabling access to the abomasum, which was then pulled to the exterior of the abdominal cavity to facilitate the administration of the drugs directly into the lumen with the aid of a 60-ml syringe and needle (40 × 12 mm.) After administration of the drug, the abomasus was placed back into position and the muscle and peritoneum were sutured with absorbable synthetic thread (Vicryl number 0). The skin was sutured with non-absorbable nylon thread (number 2–0).

Post-surgical hygiene of the wound was performed with a 1% iodine solution once a day. Antibiotic therapy was performed with benzathine penicillin 30,000 UI/kg/IM (Multibiótico, Vitalfarma) every two days for a total of three applications. Anti-inflammatory therapy was performed with flunixin meglumine at a dose of 1.1 mg/kg/IM (Banamine, MSD) for five consecutive days. These drugs were chosen due to the fact that they are not considered substrates of P-glycoprotein.

2.2.1.4. Coproparasitological evaluations and parasitological necropsy. Fecal exams were performed to evaluate the effect of the medications on the reduction in the FEC three, five, seven and nine days post-treatment using the modified McMaster method with 1:25 sensitivity and fecal cultures (Robert and Sullivan 1950). The classification of third-stage larvae was performed based on Ueno and Gonçalves (1998).

On D9, the animals were euthanized in an adequate, tranquil environment far from other animals and the lodging facility in compliance with the Guidelines for the Practice of Euthanasia of the National Animal Experimentation Control Center (CONCEA). During the pre-euthanasia procedures, care was taken to minimize suffering, fear, anxiety and apprehension. The animals were submitted to pre-anesthesia with an intramuscular injection of 1% detomidine 0.3 mg/kg, followed by anesthesia with an intravenous injection of 25% sodium thiopental at a dose of 15 mg/kg. Euthanasia was performed with an intravenous injection of potassium chloride at a dose of 40 ml/animal.

After euthanasia, the abomasum was separated using dual ligatures and the entire content was sifted (0.297 mm / Tyler 48). The solid part was fixed in 70% alcohol. The retrieval of adult and immature forms embedded in the abomasal mucosa was performed by digestion in a 3% hydrochloric acid solution at 36 °C for 24 h (Wood et al., 1995; Vercruysse et al., 2001). The total content of each abomasum was used for the examination and quantification of the parasite intensity (helminths). The count and generic identification of the helminths were performed using a stereoscopic microscope (Optika SZR-10) and the specific identification (Costa, 1982) was performed using a light microscope (Nikon Eclipse E100).

2.3. Statistical analysis

2.3.1. In vitro evaluation

2.3.1.1. Larval migration inhibition test. IVM concentrations were transformed into log (x) and the counts of the migrant larvae (survivors of the treatment) were normalized and expressed as frequency values (0 to 100%). Frequency was calculated using the following formula: percentage of migrant larvae = (n° de migrant larvae/(n° migrant larvae + *n*° non-migrant larvae)*100. The data were submitted to nonlinear regression analysis, obtaining the EC50 and a sigmoid curve of the dose-response relationship. EC50 was estimated using the following equation: $Y = 100 / (1 + 10\hat{}((\text{Log EC50} - X) * \text{Hill Slope}))$, in which Y = Bottom + (Top – Bottom) / $(1 + 10\hat{}((\text{Log EC50} - X) * \text{Hill Slope}))$, in which X = log of the concentration, Y = migrant larvae (%), Bottom = minimum percentage of migration, Top = maximum and Hill Slope = slope of the dose-response curve. After the determination of the EC50 and their respective confidence intervals for the isolates (RsHco1, FAMEZHco1 and ROHco1), the resistance factor (RF) was calculated for FAMEZHco1 and ROHco1 using the following formula: RF = EC50 of FAMEZHco1 or ROHco1/EC50 of RsHco1. The confidence intervals for the calculated EC50 values of each isolate in the different treatments were used as parameters to determine if the potentiating effect of quercetin on the anti-helminth activity of ivermectin was significant or not. Confidence intervals with any level of overlap were considered similar, therefore, no resistance-reversing effect. Non-overlapping confidence intervals were considered to be significantly different, and the potentiating effect was recognized.

For the evaluation of the potential of quercetin to inhibit the migration of third-stage larvae of *H. contortus* when used alone, the migration percentages for this treatment were compared to those for the control (0.5% DMSO) using Tukey's test for the comparison of means, considering $\alpha = 0.05$.

2.3.1.2. Adult motility test. The numbers of worms for each motility score in the wells with IVM, the combined drugs and controls were expressed as the percentage in relation of the total number of worms in the well (motility index per well for each score). The percentages of the worms with indices of motility score 3 in each well in comparison to the mean index of motility score 3 in the control were used to calculate the EC50 and generate the sigmoid curve, as performed for the larval migration inhibition test. The IVM concentrations were transformed into log (x). The six-hour evaluation time was considered for the calculation of EC50. After the determination of the EC50 for the isolates (RsHco1, FAMEZHco1 and ROHco1), the RF was calculated for FAMEZHco1 and ROHco1 using the following formula: RF = EC50 of FAMEZHco1 or ROHco1/EC50 of RsHco1). To evaluate the potential of quercetin to inhibit the motility of *H. contortus* adults when used alone, the motility score 3 indices of this treatment were compared to those obtained for the control (0.5% DMSO) using Tukey's test for the comparison of means ($\alpha = 0.05$).

2.3.2. In vivo evaluation

FEC data were transformed into log base ($y + 1$). To evaluate whether the FEC had normal distribution, the Kolmogorov-Smirnov normality test was applied ($\alpha = 0.05$). As the FEC did not present normal distribution, they were normalized (expressed as frequency

from 0, lower FEC, to 100%, higher FEC) and then, the normal distribution of the data was confirmed by the Kolmogorov-Smirnov normality test ($\alpha = 0.05$). The normalized data (expressed as frequency) were submitted to analysis of variance considering two factors: time and treatment ($\alpha = 0.05$), followed by the Bonferroni post-test ($\alpha = 0.05$).

Helminth counts were submitted to the Kolmogorov-Smirnov normality test ($\alpha = 0.05$). As non-normal distribution was demonstrated, the counts were transformed into log base ($y + 1$) and submitted to analysis of variance considering only one factor: treatment ($\alpha = 0.05$). GraphPad (Prism) version 5.03 for Windows was used for the statistical analyses.

The effectiveness of treatment regarding the reduction in the egg count in the feces was determined using the following formula: (pre-treatment mean – post-treatment mean / pre-treatment mean) x 100. Effectiveness regarding the reduction in helminth count was calculated as follows: (geometric mean of control (quercetin: group III) – geometric mean of treatments / geometric mean of control (quercetin: group III) x 100.

3. Results

3.1. In vitro evaluation

3.1.1. Effect of quercetin on larval migration and adult motility

The migration percentages in the quercetin treatment at 10^{-5} M did not differ significantly from those of the control (DMSO) ($p = 0.3350$). Quercetin also had no influence on the motility of adults ($p = 0.1885$). Therefore, when used alone, quercetin at a concentration of 10^{-5} M does not diminish the viability of *H. contortus* larvae or adults.

3.1.2. Effect of ivermectin and ivermectin + quercetin on H. contortus larvae

Quercetin did not significantly reduce the EC50 of IVM for ROHco1 or RsHco1 (the confidence intervals calculated for the EC50 of IVM and IVM + quercetin were overlapping, and were considered similar for both isolates), but led to a significant reduction in the EC50 of IVM for FAMEZHco1. Quercetin enhanced the effect of IVM on the FAMEZHco1 isolate, reducing the RF from 3.15 to 0.33 (Table 2).

3.1.3. Effect of ivermectin and ivermectin + quercetin on H. contortus adults

Quercetin did not significantly affect the EC50 for the isolates RsHco1, ROHco1 or FAMEZHco1, although it numerically increased the EC50 for FAMEZHco1 and diminished the EC50 for RsHco1 (Table 2). The RF for FAMEZHco1 was increased more than six fold. The EC50 of the IVM + quercetin combination for ROHco1 was not calculated due to the inadequacy of the nonlinear regression model in describing the behavior of the percentage of adults with motility score 3. The percentage of individuals with motility score 3 varied among the evaluation times (four, six and eight hours of incubation), with a gradual reduction in this percentage over time for FAMEZHco1 and ROHco1. The rates of individuals with motility score 3 after eight hours of incubation were lower than 50% for both isolates. The EC50 for adults was always smaller than that for larvae.

3.2. In vivo evaluation

3.2.1. Reduction in egg count in feces of animals treated through intra-abomasal route

The effectiveness of IVM at reducing the FECs in relation to the pre-treatment, ranged from 0.00 to 24.00%, reaching maximum effectiveness on the fifth day after treatment (24%; 95% CI: 0 to 90%) (Table 3). The effectiveness of IVM + quercetin ranged from 0.00 to 59.00%, reaching maximum effectiveness on D7 (59%; 95% CI: 0 to 84%). Despite the reductions, the FEC were similar among all groups at all

Table 2
EC50 for ivermectin and ivermectin + quercetin combination on third-stage larval migration test and adult motility test with three isolates of *H. contortus*: RsHco1 (sensitive to ivermectin), FAMEZHco1 (resistant) and ROHco1 (highly resistant).

Assay	Treatment /Test	RsHco1		FAMEZHco1		RF	ROHco1		RF
Larval migration inhibition test	Ivermectin	EC50 M 5.966×10^{-6}	95% CI 4.076×10^{-6}–8.734×10^{-6}	EC50 M 1.874×10^{-5}	95% CI 1.016×10^{-5}–3.457×10^{-5}	3.15	EC50 M 1.453×10^{-4}	95% CI 4.057×10^{-5}–5.202×10^{-4}	24.35
	Iver + querc.	6.234×10^{-6}	4.640×10^{-6}–8.375×10^{-6}	1.980×10^{-6}	1.457×10^{-6}–2.690×10^{-6}	0.332	1.909×10^{-4}	4.140×10^{-5}–8.807×10^{-4}	32
Adult motility test	Ivermectin	4.557×10^{-10}	1.2557×10^{-10}–1.652×10^{-9}	5.274×10^{-10}	1.225×10^{-11}–2.271×10^{-8}	1.15	5.007×10^{-8}	5.520×10^{-9}–4.542×10^{-7}	109.87
	Iver + querc.	1.375×10^{-11}	1.474×10^{-13}–1.288×10^{-9}	2.938×10^{-9}	1.505×10^{-10}–5.736×10^{-8}	6.45	–	–	–

EC50: mean effective concentration; CI: confidence interval; RF: resistance factor.

Table 3
Mean FEC and standard deviation and efficacy at reducing egg counts in feces of sheep submitted to treatments with intra-abomasal administration of ivermectin, ivermectin + quercetin and quercetin on Days 0, 3, 5, 7 and 9 post-treatment.

Parameter	Ivermectin Day 0	Day +3	Day +5	Day +7	Day +9
Mean	579.17[a]	750.00[a]	441.67[a]	783.33[a]	1640.00[a]
Stand. Dev.	893.49	1471.73	805.09	1711.48	2958.81
Efficacy (%)		0.00 (0–85)	24.00 (0–90)	0.00 (0–86)	0.00 (0–67)
	Ivermectin + quercetin Day 0	Day +3	Day +5	Day +7	Day +9
Mean	454.17[aA]	208.33[aA]	258.33[aA]	187.50[aA]	358.33[aA]
Stand. Dev.	290.73	148.88	124.16	171.57	230.58
Efficacy (%)		54.13 (0–80)	43.12 (0–71)	59.00 (0–84)	21.10 (0–64)
	Quercetin Day 0	Day +3	Day +5	Day +7	Day +9
Mean	430.56[aA]	241.67[aA]	225.00[aA]	487.50[aA]	375.00[aA]
Stand. Dev.	218.12	119.02	198.12	626.00	325.96

Means followed by the same lower case letters in a column and capital letters on the lines do not differ significantly by (Bonferroni post-test).

evaluation times ($p > 0.05$). Moreover, no significant interaction between time and treatment was found ($p > 0.05$). *Haemonchus contortus* was the most prevalent nematode in the fecal cultures (>95%).

3.2.2. Controlled anthelmintic test in animals treated through intra-abomasal route

An 8.17% reduction in the mean helminth count was found in the animals that received IVM, when compared to the animals that received quercetin (control group). However, the mean helminth count of the IVM group and the groups that received quercetin and IVM + quercetin were not significantly different ($p = 0.7878$) (Table 4). No reduction in mean helminth count was found between groups that received quercetin (control) and IVM + quercetin. All adult individuals retrieved from the abomasum were identified as *Haemonchus contortus*.

4. Discussion

When combined with IVM, quercetin enhanced the effect of this anthelmintic against larvae of *H. contortus* (FAMEZHco1 isolat: RF = 3.15), but had no effect on adults of the same parasite. The effect of quercetin on the larvae is in agreement with previous reports involving species of the genus *Haemonchus* (Bartley et al., 2009; Heckler et al., 2014) and can be attributed to modulation of resistance mechanisms, mainly P-gp (David et al., 2018). Interestingly, however, quercetin did not enhance the effect of the anthelmintic on larvae of the RsHco1 isolate, which is sensitive to IVM, or those of the ROHco1 isolate, which has a high degree of resistance to IVM (RF = 24.35). In contrast, Demeler et al. (2013) found an increase in the effectiveness of IVM against both sensitive and resistant isolates when combined with verapamil, which is another P-gp-modulating drug. Bartley et al. (2009) found that quercetin was also effective at increasing the susceptibility of first-stage larvae of *H. contortus* and *Teladorsagia circuncicta* isolates sensitive to IVM and capable of reestablishing susceptibility to resistance isolates. However, elacridar and zosuquidar, which are third-generation modulators, were not effective at increasing the susceptibility of third-stage larvae of IVM-sensitive *H. contortus*, which is in agreement with the present findings. For elacridar and zosuquidar, the efflux mechanisms by which these drugs interact are suggested to be less active in susceptible larvae than resistant larvae, which must have contributed to the effect (Raza et al., 2015).

Table 4
Number of helminths retrieved during necropsy of sheep submitted to three different treatments with intra-abomasal administration (quercetin control group, IVM and IVM + quercetin).

Treatment	Mean	Geo. mean	SD	Efficacy
Ivermectin	174.50[a]	84.92	248.04	8.17
Iver + querc.	157.67[a]	125.3	99.34	0
Querc.	100.33[a]	92.48	39.98	–

Same letters in columns denote absence of statistically significant difference in mean helminth count.

Regarding the efflux mechanism by which quercetin interacts (P-gp) (see review by Di Pietro et al., 2002), there is evidence of differences in expression levels among P-gps in the different stages of development of parasitic nematodes (Dicker et al., 2011; Sarai et al., 2013; Raza et al., 2015) and this difference is also suggested to be found between sensitive and resistant isolates (Raza et al., 2015). Therefore, these differences in expression levels and susceptibility profiles may explain the effect of quercetin in increasing susceptibility to IVM in sensitive L_1 larvae (Bartley et al., 2009), but not in sensitive L_3 larvae of *H. contortus* (as found in the present study) as well as its significant enhancement of the effect of IVM on resistant L_3 larvae of the FAMEZHco1 isolate. The absence of a modulating effect on third-stage larvae of the ROHco1 isolate also suggests the occurrence of additional resistance mechanisms (Bartley et al., 2012), which could have been important in this case.

For all isolates of *H. contortus,* adults were more sensitive to IVM than larvae, as demonstrated by the EC50. According to Demeler et al. (2014), the lower potency of IVM against L_3 may be due to a set of factors, such as the expression of receptors, the cuticular structure of the larva, the fact that larvae do not feed, the incubation conditions and *in vitro* study methods.

Quercetin did not enhance the effect of IVM on the motility of *H. contortus* adults, independently of sensitivity or resistance. The reason for this result is unclear. However, some hypotheses could be raised. Firstly, differences in the levels of P-gp expression in different development stages (Dicker et al., 2011; Sarai et al., 2013; Raza et al., 2015) may be sufficient to explain the lack of an effect. Secondly, the specificity suggested by Lespine et al. (2012) for efflux mechanisms, such as P-gps, could contribute to the inefficiency of quercetin. Considering these two hypotheses, P-gps expressed in L_3 may have greater affinity for quercetin and those expressed in adults may have less or no affinity for quercetin. A third hypothesis would be the expression of additional defense mechanisms, such as other transporters and enzymes in adults and at a greater intensity in resistant isolates (Bartley et al., 2012), particularly in response to the presence of modulators, which would not be seen in larvae. The latter hypothesis would also explain the numeric

increase in the EC50 in the FAMEZHco1 and ROHco1 isolates in comparison to that of the RsHco1 isolate. In summary, quercetin did not enhance the effect of IVM on the motility of *H. contortus* adults. Moreover, its action on larvae was influenced by the degree of resistance of the isolates, as sensitive and highly resistant isolates (ROHco1, with RF = 24.35) were not sensitive to the action of quercetin as a P-gp-modulating agent. Thus, we describe, for the first time, the influence of the development stage and susceptibility profile of helminths on the action of anthelmintics.

With regard to the evaluation of adult motility based on scores, especially when analyzing the percentage of individuals in which the score lowered from level three to lower levels, the test is quite efficient (Kotze et al., 2012). This efficiency is due to the effect of IVM on the motility of adults. IVM does not kill, but reduces motility in the central region of the nematode enough so that adults go from a motility score of 3 to 2 or 1 (Kotze et al., 2012). This reduction from a higher to lower level of motility is reasonably more important than the detection of immobile individuals and was therefore used in the present study.

The combination of IVM and quercetin administered through the intra-abomasal route had no effect regarding the reduction of the number of *H. contortus* adults. Besides, there was a moderate reduction in the number of eggs in the feces of infected animals, with no significant difference between animals treated and not treated with the combination. *H. contortus* adults were not sensitive to the combination in the *in vivo* test, which is in agreement with the results of the *in vitro* evaluation of adults with different degrees of resistance.

Several egg count reduction tests in the feces of sheep naturally infected by *H. contortus* were conducted with the combination of IVM and quercetin prior to the execution of the present study (unpublished data). During these tests, IVM was evaluated in combination with quercetin administered through the subcutaneous, intravenous and oral routes in different concentrations and with different administration intervals, including continuous infusion for 12 uninterrupted hours. The occurrence of diverse results (effectiveness from 0.0 to 62%, but with no statistical significance) regarding the reduction in the FEC in the animals treated with the combinations of IVM and quercetin was a determinant to the execution of a study using the intra-abomasal route to clarify the existence or non-existence of an enhancing effect from quercetin on the effectiveness of IVM for the treatment of animals infected by *H. contortus*.

The *in vivo* use of quercetin is limited due to the fast, efficient hepatic metabolism and renal excretion by the host, which diminishes the bioavailability of the drug. Moreover, quercetin is nearly entirely degraded by microbiota ruminal (Berger et al., 2012). Therefore, the low or absent modulating effect of quercetin in the preliminary experiments with infected sheep was initially attributed to the pharmacokinetic and pharmacodynamic characteristics of this drug. However, the final results of our experiments suggest, actually, that the absence of an effect *in vivo* was mainly due to the fact that the adults were not sensitive to the modulating action of quercetin.

Due to the low bioavailability of orally administered quercetin, different methods have been successfully employed to increase its bioavailability, such as microemulsion, dispersion in polyvinylpolypyrrolidone, complexation with lecithin and cyclodextrin (Gonzales et al., 2016) and association with lipid particles (Penalva et al., 2017). However, these strategies remain restricted to monogastric animals and further studies on ruminants are needed. Considering the pharmacokinetic and pharmacodynamic challenges of using quercetin, the intra-abomasal route was chosen for the present study as a way to ensure that quercetin and ivermectin were in direct contact with the parasite within the organism (infection site) and, therefore, subject to the chemical and physical influences of the host organism at the same time.

The difference between the *in vitro* tests performed by Bartley et al. (2009) and Heckler et al. (2014) and the *in vitro* and *in vivo* tests performed in the present study regarding the activity of quercetin as a resistance-modulating drug underscore the importance of evaluating anthelmintics and resistance modulators in different stages of development of the parasite as well as using isolates with different degrees of resistance. Based on the present findings, the effect of quercetin determined through evaluations involving a single stage of development or a single isolate with a specific degree of susceptibility can either overestimate or underestimate the modulating effect. The same is likely valid for other modulators. Therefore, future studies should prioritize structural modifications of the quercetin molecule and evaluate the influence of these changes on the bioavailability of the drug in ruminants, resistance to degradation by the ruminal microbiota and the modulation of resistance to anthelmintics in parasites in different stages of development and isolates with different degrees of susceptibility.

5. Conclusions

The enhancing effect of quercetin combined with ivermectin on the motility of larvae and adults of *Haemonchus contortus* was influenced by both the degree of resistance and the development stage of the parasite. When administered through the intra-abomasal route, the combination of ivermectin and quercetin was not effective at reducing the egg count per gram of feces or the parasite load in sheep naturally infected by ivermectin-resistant *Haemonchus contortus*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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