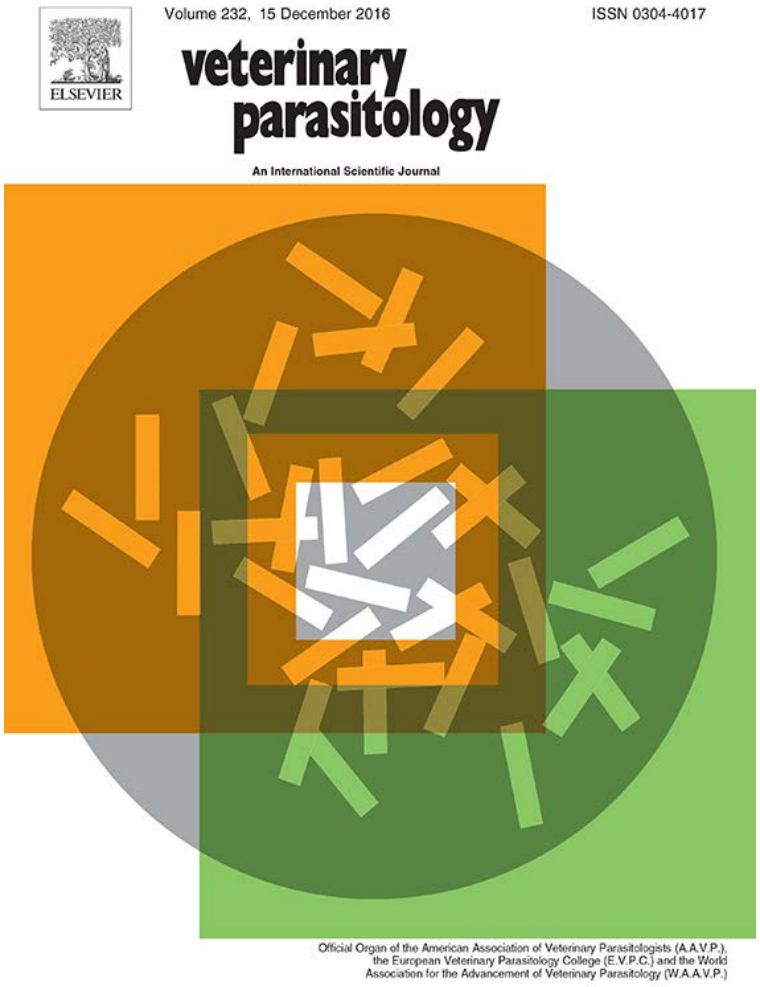

Veterinary Parasitology 232 (2016) 58–65

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Anticoccidial efficacy of naringenin and a grapefruit peel extract in growing lambs naturally-infected with *Eimeria* spp.

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ARTICLE INFO

Article history:
Received 10 July 2016
Received in revised form 7 October 2016
Accepted 8 November 2016

Keywords:
Citrus
Coccidiosis *Eimeria* spp.
Grapefruit peel extract
Naringenin

ABSTRACT

The current study aimed to determine the anti-*Eimeria* efficacy of an extract of grapefruit peels (GF) and commercial naringenin (NAR) in naturally-infected lambs, as well as the influence of these flavonoids on the oxidative status during ovine coccidiosis. Pharmacokinetic profiles were also determined. Extracts were administered *per os* to *Eimeria* naturally infected growing lambs during 90 consecutive days. The commercial anticoccidial drug toltrazuril (TTZ) was included in this trial as a standard. Twenty-four lambs were divided into four groups: NAR, lambs given a daily dose of 5 mg of a commercial naringenin extract of 98% higher purity per kg body weight; GF, lambs that recived a daily dose of 5 mg of ethanolic extract of grapefruit peels per kg body weight; TTZ, lambs treated with 20 mg of toltrazuril/kg body weight on days 0 and 15 of the experiment; and CTRL, untreated lambs that received daily dose of 30 ml of water. Daily doses of GF and NAR were dissolved in 30 ml of water and orally given to animals; whereas toltrazuril was administered as a single dose of an undiluted suspension to lambs of the TTZ group. The CTRL group received 30 ml of water; as well as the TTZ group for the period after the single dose administration. Fecal and serum samples were collected from all lambs. Anticoccidial efficacy was estimated by coprological techniques. Generation of nitric oxide levels and the antioxidant capacity of the experimental compounds were determined by the Griess and ABTS assays, respectively. The pharmacokinetic parameters of NAR and the GF extract were obtained. On day 30 post-ingestion, anticoccidial efficacy was 91.76% (NAR) and 89.65% (GF); whereas 99.63% of efficacy was achieved with TTZ 15 days after treatment. NAR, GF and TTZ significantly reduced oxidative stress in infected animals. The mean daily weight gain for each group was 122 g (NAR), 122 g (GF), 143 g (TTZ) and 98 g (CTRL). Following the oral administration of NAR and GF, values in plasma approached maximum concentrations within 2.1 to 2.5 h. In conclusion, the administration of NAR and the GF extract reduced *Eimeria* oocyst output, oxidative stress and promoted higher mean daily weight gains in infected lambs.

1. Introduction

Epidemiological studies indicate that regular consumption of citric fruits is associated with a reduced risk of coronary heart disease, inflammatory pathologies and tumor progression (Benavente-García and Castillo, 2008; Benavente-García et al., 2007). Citric flavonoids inhibit key events in angiogenesis, such as the proliferation and migration of endothelial cells (Dourado et al., 2015; Wang et al., 2015). Another flavonoid activity is to protect neurons against the damage induced by neurotoxins. There is a wide range of flavonoids, which includes: naringenin, hesperidin, catechin, epicatechin, cyanidin, wogonin, bacalein, quercetin, genistein and pelargonidin (Sun et al., 2013). Naringenin (4′, 5, 7-trihydroxyflavone) is a predominant flavanone in citrics and has a wide range of pharmacological activities. In addition to its antifibrogenic activity, it has been associated with the metastasis suppression in different types of cancer (Im et al., 2014). Naringenin is present in the orange and grapefruit peels, and has a higher potential to enter into the cell than other flavonoids, which contributes to its higher anti-inflammatory activity compared to other compounds with lower doses (Cavia-Saiz et al., 2010; Chao et al.,

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http://dx.doi.org/10.1016/j.vetpar.2016.11.009
0304-4017/

2010). It is important to point out that naringenin has been found in the brain after oral ingestion, thus potentially can exert *in vivo* anti-neuroinflammatory actions and can fight neurodegenerative diseases (Jayaraman et al., 2012; Jeon et al., 2007; Kannappan et al., 2010). Another property that this compound possesses is to block the nitric oxide production (Chang et al., 2016; Chtourou et al., 2015; Fouad et al., 2016; Ren et al., 2016). This flavanone also normalizes blood clotting factors, such as prothrombin time, fibrinogen concentration and platelet number caused by infections (Itoh et al., 2010). Naringenin exerts part of its anti-inflammatory effect by inhibiting the production of nitric oxide and the E_2 prostaglandin. Furthermore, naringenin has the ability to activate polymorphonuclear leukocytes (PMN) in order to induce cytotoxic activity (Fouad et al., 2016; Orsolić and Basić, 2005).

Animals infected with *Eimeria* spp. coccidia could not show any clinical signs, such as presenting a subclinical coccidiosis (Chapman et al., 2013). The importance of this presentation is based on that there is a continuous contamination of the animal environment; therefore, lambs may have a growing delay and feed conversion, and be predisposed to other infections. Also, when clinical signs appear, the treatment cost is often considerable (Mundt et al., 2005). Generally, it is assumed that the use of medicinal plants is safe and effective, since it has been used for many years (de Araújo et al., 2014). However, despite the use of these natural products, further research is required to carry out in order to explore the potential that these products have for diseases that have a significant health and economic impact in lambs. Additionally, the trend of using organic productions free of chemical synthesis drug residues in animal tissues and fluids, as well as, the concerns about the use of biodegradable products that do not generate antiparasitic resistance, has encouraged the study of alternative compounds for controlling diseases caused by infectious agents (Grecco et al., 2012; Haggag et al., 2011; Hernandez-Villegas et al., 2011). Therefore, the aim of this work is to evaluate the effect of a flavanone (naringenin) in order to know if it has an anticoccidial activity, and if it exerts an effect on blood concentration of reactive oxygen species and nitric oxide in naturally infected lambs with *Eimeria* spp. protozoans.

2. Material and methods

2.1. Study location

This study was performed in the Ovine Unit of the Chapingo Autonomous University, located in Texcoco, State of Mexico. The average annual temperature is 16 °C and the annual rainfall is 686 mm. Parasitological techniques and processing of the results were performed at the Parasitology Laboratory of the Faculty of Veterinary Medicine and Animal Science, UNAM.

2.2. Animals, housing, diet, management and treatments

Twenty-four crossbred female lambs with a mean body weight of 19.602 ± 0.200 kg, were naturally infected with *Eimeria* spp. and did not received any anticoccidial or antibiotic treatment during the last two months, previous to this investigation. The lambs belonged to a flock which have a history of subclinical and clinical coccidiosis. The mean weight of the two-year old lambs of this flock was 50 kg. The experiment was initiated in June, during the rainy season. Daily health observations were performed throughout the experiment.

Lambs were divided into four experimental groups (n = 6): The NAR group included lambs that received a daily dose of 5 mg/kg of body weight of a commercial naringenin extract. The GF group consisted of lambs treated with a daily dose of 5 mg per kg of body weight of the ethanolic extract of grapefruit peels. Animals belonging to group TTZ were treated with a toltrazuril suspension (Baycox® 5%; Bayer Animal Health Mexico) at a single dose of 20 mg/kg body weight according to the manufacturerís instructions. The rest of the days, animals belonging to the TTZ group received 30 ml of water. The flavonoid extracts, toltrazuril and water were administered to lambs using an oral dosing syringe (Representaciones Especiales HCR S.A. de C.V., México, México) during the 90 consecutive days of the experiment.

The CTRL group included untreated lambs that received a daily dose of 30 ml of water. The criteria for selecting the doses was based upon a previous study, which reported protective and antioxidant properties of naringenin in rats (Roy et al., 2014). Prior to administration, naringenin and the grapefruit peel extract were dissolved in 30 ml of water. Naringenin, the grapefruit peel extract, and water were administered to lambs daily before morning feeding.

The animals were approximately 60 days-old at the beginning of this study. Lambs were kept indoors on 50 cm × 150 cm × 150 cm individual concrete-floored cages fenced with cyclone mesh. Each individual animal cage was provided with a water bucket and a feed container. The diet was formulated according to NRC (2007). Animals were fed twice a day (7:00 and 13:00) and the diet consisted of (%): 14 corn silage, 20 oat hay, 35 alfalfa, and 30% of a commercial multi-particle concentrate of 16% of crude protein, as well as a commercial mineral premix (1%) with the following ingredients (g/kg): calcium (140), magnesium (10), salt (300), manganese (2.5), zinc (3), iodine (0.05), cobalt (0.125) and selenium (0.0125).

Feed samples were collected and taken to the Animal Nutrition and Biochemistry laboratory of the Veterinary Medicine Faculty of the UNAM, where a Proximal Chemical Analysis was performed. A content of 12.70% crude protein, 3.28% ether extract, 22.04% fiber and 10.72% ashes was determined, according to this analysis. Water was provided *ad libitum*.

All animals shed *Eimeria* spp. oocysts at the beginning of the study, which was demonstrated by faecal examination. All experimental procedures were conducted with the approval of the Institutional Animal Ethics Committee for the Care and Animal Use (CICUA). Daily health observations were performed throughout the experiment.

A day before the beginning of the experiment (zero day), faecal samples were obtained using polyethylene bags, which were kept in refrigeration until their analysis. Samples were examined using a faecal flotation technique in order to confirm the presence of *Eimeria* spp. oocysts; parasite load quantification was conducted using the McMaster technique (Figueroa et al., 2015). Oocyst counts were expressed as oocyst output per faecal gram (OPG), and were detected in three independent individual faecal samples through a three count average performed per sample using the McMaster technique. Before the study began, the lambs were assigned to a group based upon the faecal oocyst count (Kommuru et al., 2014).

Naringenin with a higher 98% purity was commercially obtained (Sigma-AldrichT66001, Mexico), while the grapefruit peel extract was obtained using the method proposed by Escobar (2010). We used grapefruit peel pieces (*Citrus* x *paradisi*) of 0.5 cm × 0.5 cm that were introduced into flasks. Subsequently, we added the solvent which in this case was 96% ethanol. The alcoholic extracts were evaporated in a rotary evaporator.

For allocation purposes, stratified sampling was used. Twenty-four animals were ranked from lowest to highest OPG and randomly assigned into one of the four groups (n = 6) by blocking, based on the OPG count established on zero day, before the experimental treatments.

2.3. Weight gain recording and feed intake

Each lamb was weighed at the beginning of the experiment (initial body weight) on zero day and on day 90, the last day of

the experiment. All animals were weighed individually during the morning using a suspended weighing scale (Torrey Mexico). Feed refusal was recorded by daily weighing placed and leftover feed with a digital electronic scale with 40 kg-capacity and ± 5.0 g precision (Torrey Mexico) to estimate individual feed intake.

2.4. Sampling and analysis

2.4.1. Eimeria oocyst output and species identification

In order to evaluate the efficacy of each treatment on the *Eimeria* oocyst output, faecal samples were taken at 0, 3, 7, 15, 30, 45, 60 and 90 days, and were analyzed microscopically using the faecal flotation and McMaster techniques (Figueroa et al., 2015).

Sporulation was carried out according to a previously described method (Das et al., 2015). Oocysts that were isolated from faecal material were suspended in a 300 ml solution of 2.5% potassium dichromate, and were distributed in several centrifuge tubes. The tubes were centrifuged at 671 x *g* for 5 min and the supernatant was discarded. The pellet was washed with a potassium dichromate solution, placed in a Petri dish and incubated at 28 °C under constant oxygenation. Sporulation rates were determined by counting 100 oocysts that have four clearly divided sporocysts using an ocular micrometer (reticle) (Leica Microsystems, ASPELAB, Mexico). Three repetitions were performed and counted at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 108 h (Waldenstedt et al., 2001). Subsequently, sporulation was daily monitored for two weeks. The species were morphologically determined on days 0, 15, 30 45, 60 and 90 (Reeg et al., 2005).

2.4.2. Blood sampling and processing for oxidative stress tests

Blood samples from the jugular vein (10 ml/animal) were taken in Vacutainer® vacuum tubes with EDTA as an anticoagulant agent. The tubes were transported under refrigeration to the Parasitology Laboratory of the FMVZ-UNAM in order to obtain blood serum, which was stored frozen until processing in the laboratory. Blood samplings for serum were performed at 0, 7, 14, 21, 28, 35, 42, 49, 56 and 90 days, for the determination of antioxidant capacity and nitric oxide extract generation. All sampling analyses were performed in triplicate.

2.4.3. Determination of the antioxidant capacity by ABTS assay *(2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid)*

The experiment was conducted according to the previously described methodology (Ninfali and Angelino, 2013). Initially, for testing the naringenin, the grapefruit peel extract and the toltrazuril antioxidant activities, a 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) antioxidant assay was employed (Sigma-Aldrich, Mexico). In this study, 20 μl of a serum dilution were added to 980 μl of an ABTS plus radical dilution. The absorbance was continuously measured after 7 min.

2.4.4. Determination of nitrites in serum

Total nitrites as measurement of nitric oxide were measured using the Griess reaction (de Oliveira et al., 2011). Briefly, for 200 μl of the reduced samples, 200 μl of Griess solution (sulphanilamide 2% (w/v), N-(1-naphtyl) ethylenediamine 0.2% (w/v), were added and incubated during 15 min at room temperature in the dark. The absorbance was measured at 550 nm using a UV/VIS spectrophotometer (Jenway 6305 UV/VIS, Princeton, NJ, USA). The relative nitrite concentration was calculated with the standard curve of nitric sodium. All the measurements were taken in triplicate and mean values were calculated.

2.4.5. Blood samples for pharmacokinetic studies

Five milliliters of blood were collected from the animals that were administered with 4 mg/kg of commercial naringenin or an ethanol extract of grapefruit peels in the following time intervals: 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h after the administration of the two extracts. The samples were placed at an angle of 45° degrees and left at room temperature for 30 min. Subsequently, they were centrifuged for 15 min at 2054 x *g*. Serum was obtained and placed in identified vials and frozen at −20 °C until they were processed, 8 days later.

2.4.6. Determination of pharmacokinetic variables of NAR and ethanolic extract of GF

The analytical phase was performed in the Physiology and Pharmacology Department of FMVZ, UNAM. In order to make the dilutions for the naringenin standard curve, 20 g of naringenin standard (with 98% purity) were weighed, placed in a flask, and diluted to the mark of 100 ml with deionized water (for their dissolution it was necessary to add 0.5 ml of a 0.1 N solution of NaOH prior to the addition of deionized water). Subsequently, we proceed to obtain the naringenin standard curve using the Origin Lab Pro 8© program. The difference between means and variance analysis was then evaluated. With these data, the concentration versus time plots were made, from which the pharmacokinetic values of ethanolic extract of grapefruit peels and commercial naringenin were obtained. The following pharmacokinetic values were determined: area under the curve (AUC), maximum concentration (C_{max}), maximum time (T_{max}) (hours) and half-life elimination time (T½ elim).

2.5. Data analysis

Prior to analysis, oocyst counts were converted into natural logarithms (log [OPG + 1]). All values were calculated from at least three independent experiments. The statistical model was a single factor design, with four randomized levels in complete blocks with repeated observations through time. A multivariate variance analysis was performed for repeated observations ($P < 0.0001$). Whenever the time-treatment interaction was significant, a univariate analysis of a single randomized factor design in complete blocks was performed using the Bonferroni adjustment. The Tukey test was used for multiple comparisons. The Welch test was used if variances were heterogeneous. For multiple comparisons, a Dunnett's-T test was carried out (Cervantes-Valencia et al., 2016).

The models used for this study were Proc Mixed

$$Y_{ij} = \mu + \tau_i + \delta_{j(i)} + P_k + (\tau P)_{ik} + \varepsilon_{ijk}$$

i = 1,. . .,a; j = 1,. . ., b; k = 1,..., n;where: Yij = response variable under observation, k, repetition j, treatment, i.

μ = general average.

τ_i = effect of the i-ith treatment.

$\delta_{j(i)}$ = associated error with the j-ith animal (subject) within the i-ith treatment.

P_k = effect of k-ith period.

$(\tau P)_{ik}$ = treatment x period interaction.

ε_{ijk} = random error associated with the k-ith repeated measure within the j-ith animal.

General Linear Model statistical data:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij} i = 1, 2. \ldots tj = 1, 23. \ldots r$$

where: Y_{ij} = dependent variable or response of the i-treatment and j-recurrence.

μ = is the effect of the overall average

Ti = is the effect of treatment i

ε_{ij} = is the random error, where $\varepsilon ij \sim N(0, \sigma^2)$

Table 1
Mean daily weight gain (g) 90 days after the administration of naringenin (NAR), grapefruit (GF) extracts or toltrazuril (TTZ) in *Eimeria*-infected lambs. (n = 6 lambs per group).

Experimental groups[a] (Means ± Standard Error)					
	NAR	GF	TTZ	CTRL	P value
IBW (kg)	14.67 ± 1.033	14.50 ± 1.225	14.83 ± 0.983	14.67 ± 1.033	0.995
FBW (kg)	25.67 ± 1.506[a]	25.50 ± 1.225[a]	27.67 ± 0.816[b]	23.50 ± 1.378[c]	0.033
DWG (g)	122.00 ± 0.007[a]	122.00 ± 0.000[a]	143.00 ± 0.005[b]	0.098 ± 0.008[c]	0.022

IBW, Initial body weight; FBW, Final body weight; DWG, Daily weight gain.
[abc] Different letters within a row indicate statistical significant differences (P < 0.05). For multiple comparisons, a Dunnett's-T test was done.
[a] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

Table 2
Percentage of feed refusal during the 90 days of the experiment in *Eimeria*-infected lambs treated with naringenin (NAR), grapefruit (GF) extracts or toltrazuril (TTZ) (n = 6).

	Feed refusal (%)
NAR	7.5
GF	9.6
TTZ	8.4
CTRL	6.3
P value	0.814

[1] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

3. Results

3.1. Daily weight gain and feed intake

The mean daily weight gain (g) was higher in lambs that were treated with toltrazuril (TTZ), naringenin (NAR) or grapefruit peel extract (GF) than in untreated controls (CTRL). At the end of this trial, the lambs consumed NAR and the ethanolic extract of GF, showed a daily increase in mean body weight (g) of 122 ± 0.007 and 122 ± 0.000, respectively; whereas the administration of TTZ produced an increase in the mean daily body weight of 143 ± 0.005 g. In contrast, untreated lambs gained a mean of 98 ± 0.008 g, daily (Table 1). No significant differences were, however, recorded for the percentage of the feed being consumed by lambs that received naringenin, the grapefruit peel extract or toltrazuril (Table 2).

3.2. Anti-Eimeria *spp. efficacy*

Counts of *Eimeria* OPG shed by lambs of the NAR and GF groups were similar (P > 0.05); whereas animals treated with toltrazuril (TTZ) excreted significantly (P < 0.05) less *Eimeria* OPG than the NAR, GF and CTRL groups from day 3 until day 60 (Table 3).

No significant difference (P > 0.05) was observed between the anticoccidial efficacy of the NAR and GF groups; yet these two groups significantly (P < 0.05) differed from the untreated control from day 3 until the end of the experiment. Anticoccidial efficacy was significantly (P < 0.05) higher in the TTZ group than in the NAR and GF groups. The highest anti-*Eimeria* activity was observed on day 30 for both NAR (91.76%) and GF (89.65%). On the other hand, toltrazuril-treated lambs reached an efficacy of 99.63% on day 15. On day 90, no significant difference was observed regarding OPG output among the three treated groups (Table 4).

Table 3
Effect of naringenin (NAR), grapefruit (GF) extracts or toltrazuril (TTZ) on *Eimeria* spp. oocyst output in naturally-infected lambs. (n = 6).

	Experimental groups[a] (Means ± Standard Error)				P value
Day	NAR	GF	TTZ	CTRL	
0	2408.33 ± 1265.21	2475.00 ± 1426.40	2425.00 ± 1494.91	2333.33 ± 1837.57	0.321
3	1975.67 ± 1879.51[a]	1991.00 ± 1562.29[a]	116.15 ± 250.82[b]	2325.00 ± 1022.10[a]	0.003
7	1833.33 ± 801.94[a]	1900 ± 769.41[a]	83.33 ± 12.55[b]	2808.33 ± 1374.12[c]	0.009
15	866.66 ± 651.42[a]	875.33 ± 643.90[a]	16.67 ± 2.22[b]	4541.67 ± 2207.45[c]	0.015
30	350.00 ± 162.81[a]	440.00 ± 372.95[a]	16.67 ± 6.82[c]	4250.00 ± 1410.22[d]	0.002
45	306.00 ± 300.53[a]	323.33 ± 233.15[a]	16.67 ± 3.88[b]	2291.67 ± 1523.19[c]	0.002
60	208.33 ± 144.96[a]	225.66 ± 172.30[a]	41.67 ± 12.64[b]	1741.67 ± 1344.70[c]	0.002
90	121.67 ± 14.92[a]	136.33 ± 18.83[a]	120.16 ± 17.28[a]	1025.00 ± 208.65[c]	0.002

[abc] Different letters within a row indicate statistical significant differences (P < 0.05). Dunnet's multiple comparison post-test revealed the level of significance of different treatments.
[a] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

Table 4
Anticoccidial efficacy of naringenin (NAR), grapefruit (GF) extracts or toltrazuril (TTZ) (% *versus* untreated control).

	Experimental groups[a]			P value
Day	NAR	GF	TTZ	
3	18.53[a]	17.88[a]	95.21[b]	0.0219
7	34.72[a]	21.66[a]	97.03[b]	0.0256
15	80.92[a]	80.73[a]	99.63[b]	0.0323
30	91.76[a]	89.65[a]	99.61[b]	0.0323
45	88.63[a]	88.00[a]	99.38[b]	0.0328
60	88.04[a]	87.04[a]	97.61[b]	0.0325
90	88.13	86.70	88.28	0.64

[abc] Different letters within a row indicate statistical significant differences (P < 0.05) by the multiple-comparison Dunnett's T test.
[a] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

Table 5
Speciation of *Eimeria* in stool samples of lambs treated with naringenin, grapefruit peel extract or toltrazuril.

Day	Experimental groups[a]	*Eimeria species*			
		Eimeria ovinoidalis	*Eimeria bakuensis*	*Eimeria parva*	*Eimeria pallida*
0	NAR	33	40	35	23
	GF	18	32	28	26
	TTZ	22	15	17	22
	CTRL	27	13	20	29
	SE	0.27	0.55	0.34	0.13
	P value	0.319	0.362	0.347	0.363
15	NAR	28	24	28	22
	GF	27	27	25	25
	TTZ	16	12	23	27
	CTRL	29	37	24	26
	SE	0.25	0.43	0.09	0.09
	P value	0.379	0.334	0.359	0.365
30	NAR	27	25	23	28
	GF	30	28	31	24
	TTZ	17	17	22	26
	CTRL	26	30	24	20
	SE	0.23	0.24	0.17	0.14
	P value	0.371	0.335	0.354	0.375
45	NAR	25	25	21	26
	GF	27	27	26	23
	TTZ	20	19	27	25
	CTRL	28	29	26	26
	SE	0.15	0.18	0.11	0.06
	P value	0.366	0.341	0.05	0.390
60	NAR	26	27	24	29
	GF	24	27	28	27
	TTZ	24	16	26	21
	CTRL	25	30	22	23
	SE	0.04	0.26	0.11	0.15
	P value	0.352	0.328	0.385	0.369
90	NAR	24	23	22	24
	GF	23	26	20	26
	TTZ	20	24	29	23
	CTRL	40	27	29	27
	SE	0.37	0.08	0.20	0.08
	P value	0.317	0.324	0.350	0.355

[a]NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water. Comparisons among all groups were done with the Tukey multiple comparison adjustment.

All control and treated animals excreted oocysts at each faecal examination. The highest OPG counts were recorded at the beginning of the experiment (day 0) in treated groups; whereas the untreated controls shed more *Eimeria* oocysts on day 15. At the beginning of the study (day 0), diarrhea was recorded in three lambs, that were randomly allocated in groups NAR, TTZ and CTRL. Diarrhea was not recorded in the TTZ-treated lamb on day 3. On the other hand, on days 3, 15 and 30 post-treatment administrations, diarrhea was observed in samples collected from two lambs included in the NAR and CTRL groups. Diarrhea was no longer recorded in any of the four groups from day 45 to 90. One of the 24 lambs that was randomly included in the NAR group was weak during 24 h after the treatment administration; but after this period, it was found to be in apparent good health.

The identified coccidian species in this study were: *Eimeria bakuensis*, *E. ovinoidalis*, *E. crandallis*, *E. pallida*, *E. granulosa*, *E. parva*, *E. weybridgensis*, *E. marisca*, *E. faurei* and *E. intrincata* (Table 5).

3.3. Determination of the antioxidant activity of NAR and GF

Higher antioxidant capacity ($P < 0.05$) was observed in the serum of lambs supplemented with commercial NAR, GF and TTZ *versus* untreated controls. Nevertheless, 90 days after the initiation of the trial, no differences ($P > 0.05$) were observed with respect to the antioxidant capacity in all groups (Table 6).

3.4. Measurement of nitric oxide levels

Nitric oxide (NO) production was higher in CTRL lambs compared to the other three groups. In lambs that ingested NAR and GF, with toltrazuril (TTZ), the generation of nitric oxide significantly decreased from day 14 to day 56, whereas in lambs treated with TTZ, levels of NO lowered from day 28 to 56. On day 90, NO production was similar in the four groups (Table 7).

3.5. Pharmacokinetic variables of the two groups of animals dosed with commercial NAR or ethanolic GF extract

Table 8 shows that no differences were observed between the time to reach maximum plasma concentrations of NAR and GF. Following oral administration of NAR and GF extracts, mean plasma values approached maximum concentration (C_{max}) within approximately 2.1 to 2.5 h (T_{max}), and then declined within the following 8 post-administration hours. Administration of the *Citrus* extracts resulted in a C_{max} of 1.944 ± 0.408 μg/ml for NAR and 0.8054 ± 0.118 μg/ml for GF.

Table 6
Antioxidant activity of naringenin, ethanolic extract of grapefruit peels or toltrazuril in *Eimeria*-infected lambs (MM/l, mean ± SE).

Day	Experimental groups[a]				P value
	NAR	GF	TTZ	CTRL	
0	25.26 ± 2.29	26.27 ± 1.27	28.38 ± 0.95	27.87 ± 2.19	0.365
7	40.94 ± 2.00 [a]	37.8 ± 1.45 [a]	36.31 ± 2.77 [a]	25.42 ± 0.89 [b]	0.0371
14	45.9 ± 2.16 [a]	43.54 ± 1.34 [a]	44.52 ± 2.90 [a]	25.2 ± 2.76 [b]	0.0383
21	49.43 ± 1.30 [a]	47.21 ± 0.99 [a]	50.52 ± 2.16 [a]	21.75 ± 3.25 [b]	0.0345
28	52.63 ± 1.19 [a]	50.46 ± 2.57 [a]	53.76 ± 1.54 [a]	19.4 ± 0.96 [b]	0.0245
35	54.7 ± 3.24 [a]	51.02 ± 1.31 [a]	52.28 ± 1.07 [a]	14.71 ± 1.38 [b]	0.0267
42	55.43 ± 2.59 [a]	56.74 ± 1.00 [a]	54.99 ± 2.03 [a]	16.46 ± 0.121 [b]	0.0255
49	56.37 ± 2.28 [a]	57.28 ± 1.17 [a]	53.01 ± 2.93 [a]	18.18 ± 2.41 [b]	0.0262
56	58.98 ± 2.36 [a]	59.87 ± 1.47 [a]	60.47 ± 0.94 [a]	20.52 ± 2.87 [b]	0.0249
90	36.76 ± 2.86 [a]	35.41 ± 1.15 [a]	37.3 ± 1.02 [a]	34.32 ± 1.55 [a]	0.0237

[abc] Different letters within a row indicate statistical significant differences (P < 0.05). Significance of activity of different treated groups was assessed against untreated (control) animals by Dunnett's multiple comparison tests.

[a] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

Table 7
Nitric oxide induction in *Eimeria*-infected lambs treated with naringenin, ethanolic extract of grapefruit peels or toltrazuril (nM/l, mean ± SE).

	Experimental groups[a]				
Day	NAR	GF	TTZ	NAR	P value
0	44.58 ± 1.15	45.06 ± 1.27	46.48 ± 0.71	45.26 ± 0.47	0.325
7	41.76 ± 1.32 [a]	43.98 ± 1.51	44.36 ± 0.62	45.9 ± 1.65	0.371
14	37.82 ± 0.34 [a]	39.02 ± 1.32 [a]	35.98 ± 1.12 [a]	55.41 ± 0.52 [b]	0.021
21	28.3 ± 1.30 [a]	30.65 ± 1.39 [a]	26.66 ± 0.96 [a]	51.24 ± 0.60 [b]	0.023
28	24.58 ± 0.69 [a]	26.71 ± 1.53 [a]	12.99 ± 1.67 [b]	52.94 ± 1.04 [c]	0.016
35	24.87 ± 0.44 [a]	25.8 ± 0.97 [a]	12.95 ± 1.12 [b]	55.57 ± 0.80 [c]	0.016
42	20.25 ± 0.51 [a]	22.15 ± 1.10 [a]	13.07 ± 0.61 [b]	55.67 ± 1.49 [c]	0.016
49	20.97 ± 1.16 [a]	21.67 ± 0.72 [a]	11.19 ± 1.14 [b]	52.29 ± 0.67 [c]	0.016
56	20.47 ± 1.06 [a]	20.22 ± 0.81 [a]	12.44 ± 1.64 [b]	52.76 ± 1.80 [c]	0.016
90	38.65 ± ±0.78 [a]	40.05 ± 0.73 [a]	25.44 ± 1.39 [b]	50.97 ± 1.02 [c]	0.028

[abc] Different letters within a row indicate statistical significant differences (P < 0.05). Dunnett's test was used with the GLM procedure to compare between the control and treated animals.

[a] NAR, lambs given a daily dose of 5 mg/kg BW of a commercial naringenin extract; GF, lambs that recived a daily dose of 5 mg/kg BW of an ethanolic extract of grapefruit peels; TTZ, lambs treated with 20 mg/kg BW of toltrazuril; and CTRL, untreated lambs that received a daily dose of 30 ml of water.

Table 8
Pharmacokinetic variables in *Eimeria*-infected lambs treated with naringenin, ethanolic extract of grapefruit peels or toltrazuril *per os*.

Variable	Naringenin		Grapefruit peels ethanolic extract	
	Mean	Error	Mean	Error
AUC (μg × h/ml)	10.4521	±0.425	6.1742	±0.488
C_{max} (μg/ml)	1.9400	±0.408	0.8054	±0.118
T_{max} (h)	2.1663	±0.601	2.5150	±0.417
$T½_{elim}$ (h)	2.5478	±0.240	3.6217	±0.302

AUC, Area Under Curve; C_{max}, maximum serum concentration; T_{max}, maximum time to reach Cmax; $T½_{elim}$, Elimination half-life.
Statistical analysis of plasma concentrations was carried out by means of Gaussian multi-peak regressions. C_{max} and T_{max} were obtained graphically. Values were compared by ANOVA and Bonferroni *t*-test.

4. Discussion

The result analysis of the current study showed that lambs treated with commercial NAR and GF had a reduction in faecal oocyst excretion (P < 0.05) starting three days after the ingestion of the *Citrus* extracts, in contrast to the CTRL group. It was previously demonstrated that NAR has activity against *Cryptosporidium parvum* (Mead and McNair, 2006) and *Plasmodium* spp. in a time and a concentration-dependent manner (Gboeloh et al., 2014; Inbaneson et al., 2012; Rodrigues et al., 2013). In these cases, the NAR effect on the parasite was toxic, affecting its survival and *in vitro* growth.

In the current work, the anticoccidial mechanism induced by naringenin administration was not studied. However, previous studies demonstrated that the antioxidant flavonoid xanthohumol reduced intestinal lesions and oocyst output in *Eimeria*-infected birds (Allen, 2007). The reduction in the intestinal invasion and faecal elimination of oocysts was associated with a damage of the parasite asexual stage. It was previously published (Landi-Librandi et al., 2012) that the favorable effects exerted by polyphenols in parasitized animals are caused by a reduction in the generation of oxidative stress as a defense mechanism of the host (Erlund et al., 2001). Flavonoids of *Ageratum conyzoides* showed a reduction in the oxidative stress caused by *E. tenella* in poultry that it's ingested at a rate of 500–1000 mg/kg. Bird production parameters, such as feed conversion and weight gain increased; whereas the intestinal lesions caused by *Eimeria* decreased (Nweze and Obiwulu, 2009). Moreover, the efficacy of toltrazuril in *Eimeria*-infected housed lambs has been demonstrated in several previous studies (Balicka-Ramisz, 1999; Diaferia et al., 2013; Mundt et al., 2009).

Previous studies in birds infected with *Eimeria* spp. that ingested antioxidant plant extracts, such as *Tulbaghia violacea* (35 g/kg), *Vitis vinifera* (75 mg/kg) and *Artemisia afra* (150 mg/kg); showed an increased in feed conversion, similar to the one obtained with a commercial anticoccidial drug (Naidoo et al., 2008). In that experiment, the authors demonstrated that NAR has antioxidants, which

interfere directly with free radicals and therefore restore the oxidant/antioxidant balance, in order to reduce injuries caused by *Eimeria* spp, as lipid peroxidation changes enzyme activity and amino acid structure, therefore causing cell toxicity (Faine et al., 2011).

In the present study, NAR, GF and TTZ showed an influence on the oxidative status. Previous studies (Fouad et al., 2016; Im et al., 2014; Sachdeva and Flora, 2014), indicate that naringenin is rich in OH groups providing hydrogen atoms to free radicals that block the oxidative chain reaction. Moreover, it has been stated that naringenin owes its antioxidant properties to the presence of phenolic rings, which act as electron traps for scavenging peroxiradicals and superoxide anions (Jeon and Lee, 2014; Jeon et al., 2007). Currently, the use of plant extracts with antioxidant potential has gained particular importance in view of the restriction on the use of synthetic compounds against coccidial infections, due to the emergence of drug resistance and drug residues (Haggag et al., 2011). Therefore, the use of natural antioxidants could rectify the difficulties associated to the use of synthetic drugs, since they are not only natural products, but also possess new molecules against which resistance genes have not been selected yet. The use of natural antioxidants could also meet the growing interest of consumers on the safety of animal origin food products. As far as we know, this is the first study that demonstrates the antioxidant activity of toltrazuril.

The pathogenic and low-pathogenic *Eimeria* species that were identified and quantified in this trial showed no difference in numbers, except on day 90, when the *E. ovinoidalis* percentage was higher ($P < 0.05$) in CTRL lambs compared to the other three groups.

Both naringenin and the ethanolic grapefruit peel extract, reduced nitric oxide (NO) generation in lambs infected with *Eimeria* spp. of the present study. The precise mechanism by which naringenin or the grapefruit extract decreases NO production was not investigated; however, it does not discard the possibility that the regulatory naringenin activity is a direct consequence of the parasitic load reduction, which ultimately result in the induction of less inflammatory mediators. That is, the resting macrophages lack of iNOS in order to initiate NO synthesis. The iNOS expression can be initiated by various stimuli, including pathogens or molecules associated to pathogens (Henard et al., 2014). Despite its function to eliminate the infectious agent, an excessive NO production can also be detrimental to the host (Jayaraman et al., 2012). It has been shown that incubation of avian macrophages with crude *E. tenella* merozoite lysates produce high levels of NO, apparently regulated by the iNOS expression, which associated with severe inflammation, damage to intestinal epithelial cells and an increased invasion of *E. tenella* (Chow et al., 2011). The main target of nitrosative stress is the depletion of reduced glutathione through its nitrosylation, which exacerbates the oxidative stress and causes the cells to be more susceptible to diseases (Kannappan et al., 2010). Although, there is no information from previous studies, regarding the activity of naringenin on NO generation in ruminant coccidiosis, there are sufficient published statements that support its effectiveness in reducing NO levels (Amira et al., 2008; Chao et al., 2010; Jayaraman et al., 2012; Vafeiadou et al., 2009).

With respect to the lamb mean daily weight gain, it was observed that the animals ingesting naringenin and grapefruit, significantly gained more weight than untreated controls. However, the most significant mean daily weight gain was recorded in lambs that received toltrazuril. This result is inconsistent with parasitological helminth studies, since the administration of citric pulp to sheep infected with gastrointestinal nematodes had no effect on weight gain; however, it improved the final body condition of sheep (Nordi et al., 2014). Our results agree with previous reports, which demonstrated that daily weight gains was higher in *Eimeria*-infected sheep treated with toltrazuril (Le Sueur et al., 2009).

The anticoccidial efficacy produced by the naringenin (NAR) and the grapefruit peel (GF) extracts cannot be considered high or viable from a commercial perspective, since it was lower than the one observed after toltrazuril administration. One possible cause for this low efficacy might be due to the pharmacokinetic profile of the extracts. In the respective experiment, it was shown that following oral administration of NAR and GF extracts, mean plasma values approached maximum concentration (C_{max}) within approximately 2.1 to 2.5 h (T_{max}), and then declined within the following 8 post-administration hours. This result is similar to a previous finding (Ma et al., 2006), in which 30, 90 or 270 mg/kg of naringenin were orally administered to Wistar rats and reached maximum concentrations at 0.5, 2 and 2 h, respectively.

Administration of the *Citrus* extracts resulted in a C_{max} of 1.944 ± 0.408 μg/ml for NAR and 0.8054 ± 0.118 μg/ml for GF in this experiment. The latter result is similar to a previous study (Sun et al., 2013), that reported a C_{max} of 0.77606 μg/ml in mice following naringenin administration. We speculate that the difference regarding the C_{max} of NAR in the current trial, might be due to the animal model used, as gastrointestinal metabolism is different in ruminant species (Koritz, 1983). Hence, it is suggested to perform further pharmacokinetic studies using different dosing protocols with NAR and GF in order to elucidate if these flavonoids are time-dependent and if high plasma concentrations are required to increase parasite exposure and consequently, exert the anticoccidial action.

5. Conclusions

Under the conditions of the current trial, the oral administration of a commercial extract of naringenin or a grapefruit peel ethanolic extract to *Eimeria*-infected lambs reduced the parasite load, influenced oxidative stress and increased weight gain in contrast to untreated controls.

Acknowledgments

The authors are grateful to Prof. Irene Cruz Mendoza and Mateo Salazar Islas for technical assistance in stool and blood sampling, as well as for carrying out blind copromicroscopical techniques.

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