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Pharmacological characterization of geraniol in sheep and its potential use in the control of gastrointestinal nematodes

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ABSTRACT

Geraniol (GNL) was effective against gastrointestinal nematodes *in vitro*; nevertheless, the anthelmintic effect of phytochemicals combined with synthetic drugs has been little explored *in vivo*. This article characterized *in vitro / in vivo* the pharmacological features of GNL in sheep as well as its pharmacokinetic interaction with albendazole (ABZ). Additionally, the *in vivo* efficacy of GNL against *Haemonchus contortus* was evaluated in lambs. Liver microsomes from lambs were incubated in the absence or presence of GNL to analyze CYP1A1, CYP1A2 and FMO metabolic pathways. The effect of GNL on the hepatic sulfoxidation and sulfonation of ABZ and the ruminal sulforeduction of albendazole sulfoxide (ABZSO) was assessed. The *in vivo* pharmacokinetic interaction of ABZ and GNL was evaluated in lambs. The effect of GNL on the fecal egg count was evaluated in lambs infected with a resistant isolate of *H. contortus*. In sheep liver microsomes, the presence of 2 mM GNL reduced the CYP1A1, CYP1A2 and FMO pathways by 77.9, 90.8 and 84.5%, respectively, with respect to control ($P < 0.05$). In the presence of 2 mM GNL, the ABZ sulfoxidation decreased from 114.4 ± 8.49 (control) to 50.24 ± 11.1 nmol/min. mg, and $ABZSO_2$ production decrease from 0.52 ± 0.14 to 0.09 ± 0.03 nmol/h.mg. No changes in the pharmacokinetic behavior of ABZ were observed in the presence of GNL. The *in vivo* efficacy of four doses of GNL was 40.5%. These findings highlight the importance of integrated *in vitro / in vivo* pharmaco-parasitological studies to develop new pharmacological tools for controlling gastrointestinal parasites.

1. Introduction

Gastrointestinal nematodes may cause subclinical and, in some cases, serious clinical infections in ruminants in many countries around the world. Therefore, efficient deworming programs are required to prevent production losses in livestock (Kaplan, 2020). Over the last 50 years, nematode control was mainly accomplished using repeated treatment with synthetic anthelmintic drugs (Gilleard et al., 2021). However, the sustainability of this approach is at risk due to the rapid spread of drug-resistant parasite populations (Kaplan & Vidyashankar, 2012). Nowadays, there is a need to develop novel strategies for extending the lifespan of anthelmintic drugs. The combination of compounds with different mechanisms of action was proposed as an important method for delaying resistance development (Lanusse et al., 2018).

While plant components have been used as dewormers since ancient times, their *in vitro* and *in vivo* activity under laboratory and farming conditions has been studied only in the last 10–15 years (Peña-Espinoza et al., 2018). Consequently, bioactive phytochemicals are now being evaluated as possible parasite control methods in livestock (Katiki et al., 2019; Miró et al., 2020a). Geraniol (GNL) (3,7-dimethylocta-trans-2, 6-dien-1-ol) is a monoterpene with abundant presence in essential oils extracted from aromatic plants, such as rose, lavender, and lemongrass (Lapczynski et al., 2008). This compound is widely known for possessing very interesting pharmacological features, including antimicrobial, anti-inflammatory, antioxidant, neuroprotective, and anticancer properties (Pavan et al., 2018). The potential therapeutic effects of GNL may have significant clinical implications, since this phytochemical is classified as generally-recognized-as-safe (GRAS) by the Food and Drug Administration (Lapczynski et al., 2008).

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The anthelmintic activity of GNL, either isolated or as a part of essential oil, has been previously examined under *in vitro* conditions. The *in vitro* mortality effect of GNL against the model *Caenorhabditis elegans* was demonstrated with a LC_{50} of 14 μg/mL (Abdel-Rahman et al., 2013). Furthermore, GNL was found to reduce larval motility of *Haemonchus contortus, Trichostrongylus axei* and *Teladorsagia circumcincta* by 82%, 90% and 94%, respectively (Helal et al., 2020). These promising findings highlight the pharmacological challenge of studying the potential of GNL as a useful tool for treating gastrointestinal parasites in ruminants.

In addition to its own anthelmintic activity, GNL may be combined with synthetic anthelmintics to enhance their anthelmintic action. As a result of pharmacokinetic and/or pharmacodynamic interactions, bioactive phytochemicals may increase the efficacy of existing anthelmintic drugs (Miró et al., 2020b). Albendazole (ABZ), a member of the methylcarbamate benzimidazole family of anthelmintics, is effective against gastrointestinal nematodes, cestodes and adult liver flukes older than 12 weeks in ruminants (Lifschitz et al., 2017). ABZ is extensively metabolized in the liver by flavin-containing monooxygenase (FMO) and cytochrome P450 (CYP) families. Both enzyme pathways are involved in the parent drug's two-step sulfoxidation into ABZ sulfoxide (ABZSO) and sulfone ($ABZSO_2$) metabolites (Galtier et al., 1986; Lanusse & Prichard, 1993; Virkel et al., 2004). Despite these virtually reversible metabolic reactions taking place within the organism, the aforementioned sequential hepatic metabolism of ABZ results in a decrease in its anthelmintic activity, to some extent. Consequently, interference with liver metabolism may cause changes in the kinetic behavior of benzimidazole metabolites (Lanusse & Prichard, 1993). *In vitro* data showed that GNL strongly inhibited CYP2B6 hydroxylase activity in a competitive manner (Seo et al., 2008). The potential clinical use of GNL appears as a promising alternative; however, deeper pharmacological studies are required in ruminants to design appropriate GNL formulations and rational therapeutic protocols. The aim of this work was to characterize *in vitro* and *in vivo* the pharmacological features of GNL in sheep and its pharmacokinetic interaction with ABZ. Additionally, the *in vivo* effect of GNL on the fecal egg count (FEC) was evaluated in lambs infected with the abomasal nematode *H. contortus*.

2. Materials and methods

2.1. Study of hepatic metabolic interactions

Liver microsomes were prepared from four healthy Corriedale lambs (8 months old) obtained from a local abattoir. Samples (approximately 20 g) of liver parenchyma (caudate process) were taken and cleaned with ice-cold KCl (1.15%) to remove hemoglobin; then they were stored in aluminum foils and frozen in liquid N_2. Tissue samples were brought to the laboratory for subsequent procedures. The preparation of liver microsomes was performed by differential centrifugation following previously reported procedures (Maté et al., 2008). An aliquot of each microsomal preparation was used for protein measurement by the method described by Lowry et al. (1951). Microsomal suspensions were stored at −70°C until use in the different biotransformation assays. The N-dealkylation rates of 7-ethoxyresorufin (7-ERR) and 7-methoxyresorufin (7-MRR) were measured as indicatives of CYP1A1- and CYP1A2-dependent metabolism, respectively (Pegolo et al., 2010). The FMO-dependent metabolism was evaluated by the measurement of the rate of N-oxidation of benzydamine (BZ) (Capolongo et al., 2010). A typical reaction mixture contained (in a final volume of 0.5 mL): phosphate buffer 0.1 M (pH 7.4) consisting of 0.8 mM EDTA, 5 mM $MgCl_2$, 0.32 mM $NADP^+$, 6.4 mM glucose-6-phosphate, 1.25 U of glucose-6-phosphate dehydrogenase, 0.2 mg/mL of microsomal protein and the respective substrates (1 μM 7-ERR, 5 μM 7-MRR and 50 μM BZ). The aforementioned substrates were incubated alone (control assays) or in the presence of 0.2 and 2 mM of GNL. Blank incubations were prepared with all components of the reaction mixture except for the NADPH-generating system. These incubations were used as controls for possible non-enzymatic drug conversion, which was subtracted from the assays performed in the presence of NADPH. All metabolic reactions were performed in glass vials placed in a water-shaking bath (Yamato Scientific Co., Tokyo, Japan) and incubated at 37°C for 5 min. Assays were stopped by the addition of 0.3 mL of ice-cold acetonitrile (CYP-dependent metabolism) or 0.5 mL of methanol (FMO-dependent metabolism) and immediately stored at –20°C until analysis.

The interference of GNL as a potential inhibitor of ABZ metabolism was assessed by evaluating the formation of ABZSO and $ABZSO_2$ in sheep liver microsomes. Reactions were prepared as previously described. ABZ (50 μM) was incubated with 1 mg of microsomal protein for evaluating ABZ S-oxygenation. For the ABZSO sulfonation assay, 2 mg/mL of microsomal protein and 50 μM ABZSO was used as substrate. Incubations were performed in the absence (control assays) or in the presence of 2 mM GNL. The reaction mixture was prepared in glass vials and incubated during 15 and 60 min for the ABZ S-oxygenation and sulfonation, respectively, at 37°C in a water-shaking bath under aerobic conditions. Assays were stopped by adding 0.2 mL of ice-cold acetonitrile and immediately stored at –20°C until analysis by high performance liquid chromatography (HPLC).

2.2. Evaluation of pharmacokinetic interactions between GNL with ABZ

2.2.1. In vitro ruminal incubations

ABZSO sulforeduction was assessed by the amount of ABZ formed in active rumen content. The potential interference of GNL in the ruminal metabolism of ABZ was studied using the technique described by Virkel et al. (2002). Briefly, aliquots (2 mL) of rumen content from three lambs were incubated with 50 μM ABZSO alone (control assays) or in the presence of 0.52 and 5.19 mM GNL. The concentration of 5.19 mM reflects the theoretical ruminal concentration obtained after oral administration of GNL at the dosage of 100 mg/kg. Unfortified rumen content samples with the same volume of methanol were incubated as blanks. Incubation tubes with rumen content were shaken in a water bath at 38°C under anaerobic conditions for 30, 60 or 240 min. After the incubation periods, each sample was immediately frozen at −20°C until analysis.

2.2.2. In vivo pharmacokinetic interactions

The pharmacokinetic interaction of GNL and ABZ after oral administration of both compounds was studied in a randomized, two-period cross-over study in six healthy Corriedale lambs (mean average body weight; 23 kg) with a washout period of 2 weeks before the second treatment. Animals were placed in a paddock, where they were fed with commercial concentrates and provided with water *ad libitum*. They were assigned to two (2) experimental groups. Group A received ABZ (Baxen®, Tecnofarm, Argentina) commercial suspension (5 mg/kg, orally). Group B received ABZ, at the same therapeutic dosage, and GNL (Euma, Argentina) (100 mg/kg, two oral doses administered at -1 and 9 h post ABZ administration). After the washout period, group A was treated with GNL and group B with ABZ by the same route and using the same dosage as in phase 1. GNL was diluted in Tween 80:chitosan 1% (1:6) at a final concentration of 22% of GNL. Jugular blood samples (2 mL) were collected into heparinised vacutainer tubes at 1, 3, 6, 9, 12, 18, 24, 30, 36, 48 h post-treatment. Blood samples were centrifuged at 2000 g for 15 min; the recovered plasma was kept in labeled vials and stored at −20°C until the analysis of ABZ metabolites and GNL plasma concentrations

2.3. In vivo effect of GNL on the FEC

For this test, GNL was encapsulated into yeasts, following an adaptation of the procedures described by Bishop et al. (1998) and Nelson et al. (2006). Briefly, pure GNL, dry active yeast *(Saccharomyces cerevisiae)* particles (YP), and distilled water were mixed in a 1:2:4 ratio and

incubated for 24 h in an orbital shaker at 38°C. The yeast cells containing encapsulated GNL were harvested by centrifugation (8000 g) during 15 min. The pellet was aliquoted in vials containing 3 g of GNL each. For the treatment, the yeast pellet was resuspended in 4 mL of sesame oil and administered orally to lambs. The trial involved six Corriedale lambs (average body weight 27 kg) artificially infected with 2000 larvae of a highly resistant isolate of *H. contortus* to ivermectin. This isolate was obtained in our Lab (Maté et al., 2018) and is maintained under laboratory conditions through successive passages in parasite-free lambs. At the beginning of the trial (30 days after experimental infection), the average of eggs per gram of feces (epg) was 7100 ± 9200 epg. The analysis of epg, was performed using the modified McMaster technique with a sensitivity of 10 epg (Roberts & O'sullivan, 1950). Lambs received GNL (100 mg/kg, four oral doses administered every 24 h). Jugular blood samples were collected and processed in the same way as in the previous trial, at 1, 9, 24, 33, 48, 57, 72, 81 and 96 h post-treatment. The effect of GNL was determined by collecting fecal samples on days −1, 7 and 14 post-treatment to evaluate the fecal egg count reduction (FECR), according to the following formula (McKenna, 1990):

$$FECR\ (\%) = 100\ x\ \left(1 - \frac{T2}{T1}\right)$$

where T1 are the arithmetic mean epg counts on days -1 and T2 are the epg counts at 7 and 14 post-treatment. The 95% confidence intervals were calculated following Coles et al. (1992).

2.4. Chromatographic analysis

The quantification of ABZ and its metabolites in microsomal incubations, plasma samples and incubated ruminal contents were performed following previously reported procedures (Alvarez et al., 2008; Lanusse et al., 1992; Virkel et al., 2004). Oxibendazole (OBZ), as internal standard, was added to each sample. A liquid-liquid extraction with acetonitrile was performed for microsomes and ruminal contents, whereas a solid phase extraction with C18 cartridges (RP-18, 100 mg, Strata, Phenomenex, CA, USA) was done for plasma samples. Experimental samples were analyzed by HPLC (Shimadzu) using a Kromasil C18 (5 µm, 250 mm x 4.60 mm, Eka Chemicals, USA) reverse-phase column with a UV detector set at 292 nm, as described by Alvarez et al. (2008) and Virkel et al. (2004).

Resorufin (RES), the metabolite formed from CYP1A1 and CYP1A2 metabolic pathways, was quantified in an HPLC system coupled with a Kromasil C18 (5 µm, 250 mm x 4.60 mm, Eka Chemicals, USA) reverse-phase column and a Shimadzu RFXL fluorescence detector set at 530nm (excitation) and 580 nm (emission) wavelengths (Pegolo et al., 2010). The concentrations of BZ N-oxide were measured using HPLC, with fluorescence detection set at 303nm (excitation) and 350 nm (emission) (Capolongo et al., 2010).

A liquid-liquid extraction was performed to determine GNL concentrations from the plasma and yeast samples. An aliquot of 0.5 mL (plasma) or 100 mg (yeast) was mixed with 0.5 mL of cold acetonitrile and agitated for 10 min in TissueLyser II (Qiagen, Germany). The mixture was centrifuged at 15000 g at 4°C for 10 min. The supernatant was transferred into a tube and 100 µL was injected into the HPLC system. GNL was analyzed by HPLC following the technique described by Pavan et al. (2018), with minor modifications. Briefly, the UV detection was set at 210 nm and the mobile phase consisted of a mixture of water and acetonitrile (47/53%) at an isocratic flow of 1.4 mL/min.

Calibration curves were prepared using the least squares linear regression analysis (Instat 3.00, Graph Pad Software, Inc., San Diego, USA) of HPLC peak area ratios of analytes/internal standard (ABZ metabolites) or external standard (resorufin, BZ N-oxide and GNL) and nominal concentrations of spiked samples. Square correlation coefficients (R^2) were between 0.995 and 0.999 and lack-of-fit tests were also performed to confirm the linearity of the regression line of each analyte. The concentrations in the experimental samples were determined following interpolation of peak area ratios of analytes/internal or external standards into their calibration straight lines.

2.5. Data analysis

For the evaluation of specific metabolism CYP1A1, CYP1A2 and FMO, the metabolic rates are expressed in ρmol of metabolic products formed per minute per mg of microsomal protein (ρmol/min.mg). In the incubations of microsomes with ABZ or ABZSO, metabolic rates are expressed in nmol of metabolic products formed per hour per mg of microsomal protein (nmol/h.mg). The concentrations of the ABZ parent drug formed after the ABZSO incubation alone or in the presence of GNL are expressed as nmol/mL of ruminal content (± SD). The plasma concentration versus time curves obtained after treatment of each individual animal with ABZ alone or ABZ-GNL were fitted with the PK Solutions 2.0 computer software (Ashland, OH, USA). Pharmacokinetic parameters were determined using a non-compartmental model method (Gibaldi & Perrier, 1982). ABZSO and $ABZSO_2$ plasma concentrations and all the estimated pharmacokinetic parameters are reported as mean ± standard deviation (SD). The statistical analysis was performed using the Instat 3.0 software (GraphPad Software, CA, US). Depending on the experiment, statistical comparisons of drug concentrations, metabolic rates, pharmacokinetic parameters and epg counts were statistically compared using Student t test, Mann-Whitney test, ANOVA or Kruskal–Wallis test. Differences were considered statistically significant at P values below 0.05.

3. Results

3.1. Study of metabolism interactions in liver

Biotransformation assays were performed to estimate the inhibition of specific metabolic pathways by GNL (Fig. 1). The CYP1A1 pathway was evaluated by means of 7-ERR O-deethylase activity (EROD). In control assays EROD activity was 259.8 ± 28.5 ρmol/min.mg of microsomal protein. The addition of 0.2 and 2 mM of GNL decreased the rate of this metabolic reaction to 152.0 ± 38.2 and 57.5 ± 8.5 ρmol/min.mg, respectively ($P < 0.05$ and $P < 0.01$) (Fig. 1A). The CYP1A2 pathway (Fig. 1B) was tested by the measurement of 7-methoxyresorufin O-demethylase (MROD) enzyme activity. Compared to control incubations, MROD only decreased significantly ($P < 0.05$) after the addition of 2 mM of GNL (from 67.9 ± 20.1 to 6.26 ± 1.56 ρmol/min.mg). Similarly, the FMO-dependent metabolism was also affected in presence of GNL 0.2 mM and 2 mM; BZ N-oxidase activity resulted 65.5 ± 13.6 and 84.5 ± 4.84 % lower ($P < 0.05$, $P < 0.01$) compared to that observed in the absence of the monoterpene (Fig. 1C).

Liver microsomes were able to metabolize ABZ and ABZSO mainly to their respective S-oxidized metabolites. Fig. 2 shows the rate of production (nmol/h.mg) of ABZSO and $ABZSO_2$ in the absence and presence of GNL. In the presence of 2 mM GNL, the metabolic rate of ABZSO production decreased from 114.4 ± 8.49 (control incubations) to 50.24 ± 11.1 nmol/h.mg ($P < 0.001$). Similarly, the sulfonation of ABZSO to $ABZSO_2$ also decrease in the presence of GNL. The rate of $ABZSO_2$ production was 0.52 ± 0.14 nmol/h.mg in control assays and dropped to 0.09 ± 0.03 nmol/h.mg in the presence of GNL ($P < 0.01$).

3.2. Evaluation of pharmacokinetic interactions between GNL with ABZ

3.2.1. In vitro ruminal incubations

ABZ was the metabolic product formed after the ruminal sulforeduction of ABZSO under anaerobic conditions. The presence of GNL did not affect the formation of ABZ. Table 1 shows the concentrations of ABZ, formed from ABZSO, in rumen content incubations with or without

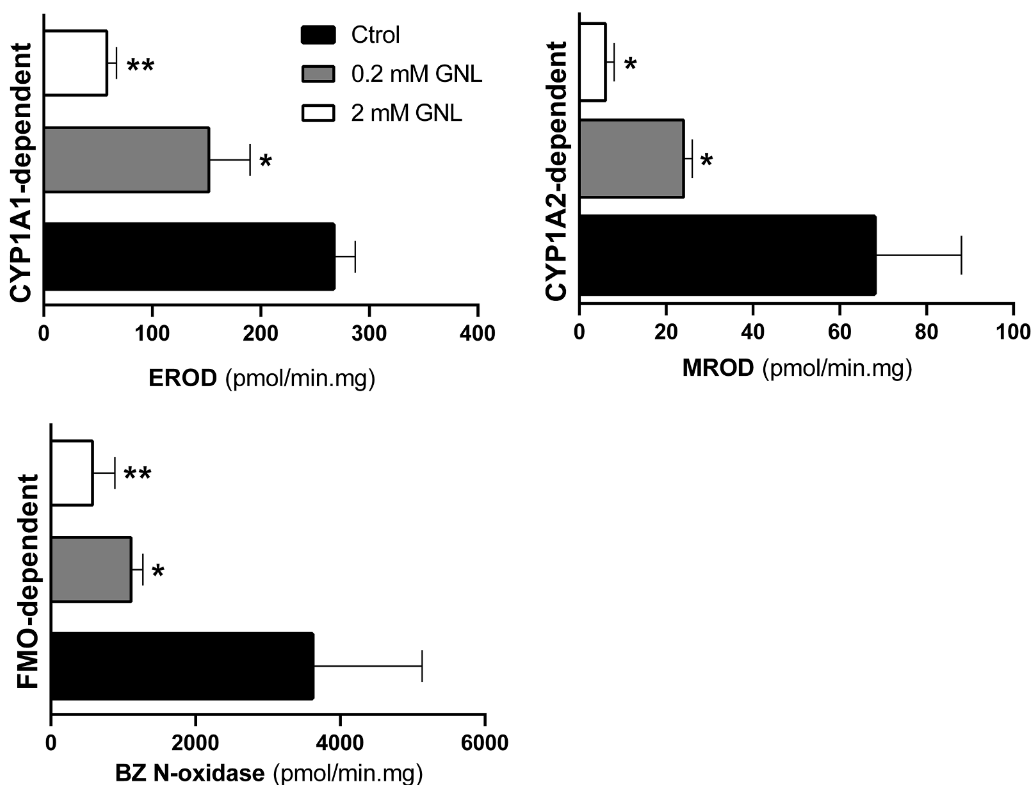

Fig. 1. Effects of geraniol (GNL) on cytochrome P450 (CYP) 1A1 (A.), 1A2 (B.) and flavin-containing monooxygenase (FMO) (C.) specific metabolic pathways in sheep liver microsomes. The formation of resorufin (RES) and benzydamine (BZ N-oxide) was quantified. Data (pmol/min.mg of microsomal protein) are expressed as mean (±SD). Incubations were performed in duplicate with liver microsomal preparations from four lambs. Incubations are significantly different from control at $^{*}P < 0.05$ and $^{**}P < 0.01$.

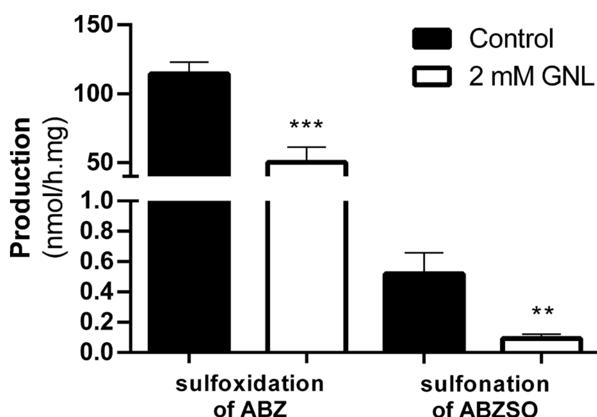

Fig. 2. Effect of geraniol (GNL) on the *in vitro* sulfoxidation of albendazole (ABZ), to albendazole sulfoxide (ABZSO) and sulfonation of ABZSO to albendazole sulfone ($ABZSO_2$) by sheep liver microsomes. Metabolic rates (mean ± SD) are expressed as nmol product formed per h.mg of microsomal protein. Incubations were performed in duplicate with liver microsomal preparations from four lambs. Incubations are significantly different from control at $^{**}P < 0.01$ and $^{***}P < 0.001$.

0.52 and 5.19 mM of GNL.

3.2.2. In vivo pharmacokinetic interactions

Both ABZSO and $ABZSO_2$ metabolites were detected in plasma after the oral administration of ABZ, either alone or co-administered with GNL. The mean plasma concentrations of ABZSO and $ABZSO_2$ in the two groups are shown in Fig. 3A and B, respectively. The comparative pharmacokinetic parameters obtained after the administration of ABZ either alone or co-administered with GNL in lambs are shown in Table 2. Concomitant administration with GNL did not change the plasma concentrations of ABZ metabolites with respect to the control group. GNL was not detected in plasma between 1 and 9 h post-administration of the first doses. After 1 h of the administration of second dose of GNL, the mean plasma concentration was 3.04 ± 1.33 μg/mL.

Table 1
Effect of 0.52 and 5.19 mM Geraniol (GNL) on the ruminal sulforeduction of albendazole sulfoxide (ABZSO) into albendazole (ABZ) under anaerobic conditions. Values were compared with those measured after incubations with ABZSO alone (control assays) for each of the incubation times. Data are expressed as mean (±SD). Incubations were performed in duplicate with liver microsomal preparations from three lambs.

Incubation Time (min)	Incubation conditions	ABZ (nmol/mL)
30	ABZSO alone	20.8 ± 15.1[a]
	ABZSO + $GNOL_{0.52}$	28.3 ± 16.6[a]
	ABZSO + $GNOL_{5.19}$	27.5 ± 7.7[a]
60	ABZSO alone	24.7 ± 11.6[a]
	ABZSO + $GNOL_{0.52}$	34.2 ± 22.2[a]
	ABZSO + $GNOL_{5.19}$	31.1 ± 6.8[a]
240	ABZSO alone	29.2 ± 25.1[a]
	ABZSO + $GNOL_{0.52}$	51.6 ± 35.7[a]
	ABZSO + $GNOL_{5.19}$	54.3 ± 22.3[a]

*Different lowercase letters between incubation conditions indicate statistically different values.

3.3. In vivo effect of GNL on FEC

GNL was encapsulated in yeast in an attempt to increase its contact with gastrointestinal nematodes. The concentration of GNL in the administered doses was measured by HPLC. The efficiency of the encapsulation process of GNL within the yeasts was 99.5 ± 20.7 %. GNL was detected in plasma only at 1 h post-treatment of each daily administration. The mean GNL plasma levels were between 0.71 ± 0.5 and 1.42 ± 2.06 μg/mL. The FEC and the results of the FECR are shown in Fig. 4. The administration of four doses of GNL did not significantly decrease the fecal egg counts at 7 and 14 days after the administration of first dose. The mean epg counts (± standard deviation) at day -1, 7 and 14 were 7200 ± 9200, 6930 ± 6500 and 4223± 2726.

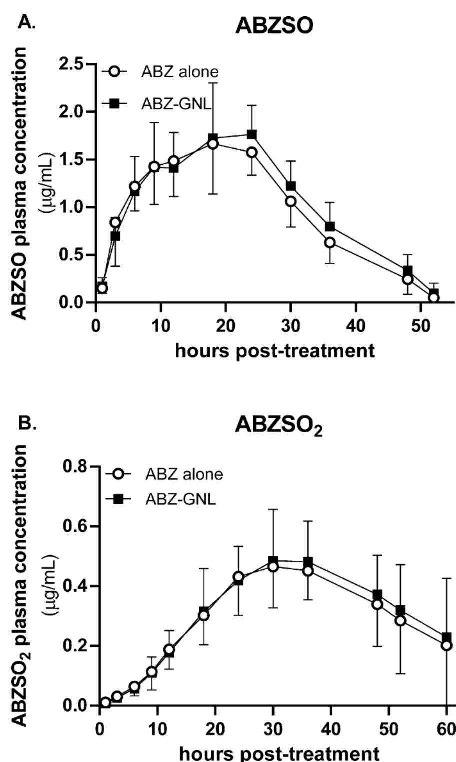

Fig. 3. Plasma concentration profiles of (A.) albendazole sulfoxide (ABZSO) and (B.) albendazole sulfone ($ABZSO_2$) after oral administration of albendazole (ABZ) parent drug at its recommended dose (5 mg/kg) either alone or co-administered with GNL (Geraniol, two doses of 100 mg/kg each at -1 and 9 h post-administration of ABZ) to lambs (n = 6).

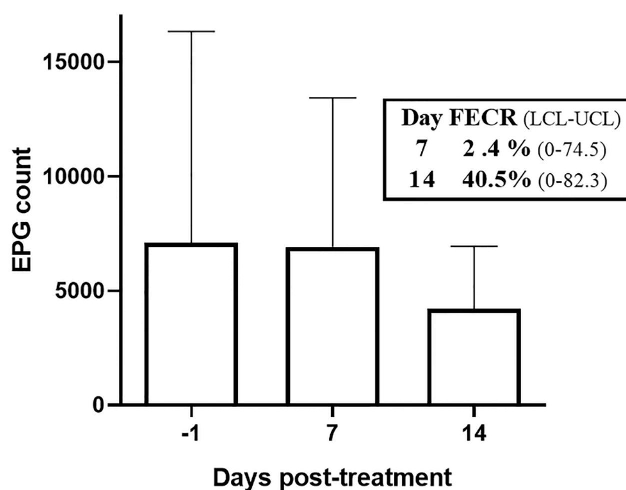

Fig. 4. Egg per gram (epg) counts (mean ± standard deviation) at -1, 7 and 14 days post-treatment with geraniol (four oral doses administered every 24 h at 100 mg/kg) to Corriedale lambs artificially infected with a resistant strain of *H. contortus*. The insert shows the reduction percentages of fecal egg counts (FECR) with their lower and upper confidence intervals at 95% (LCL-UCL).

Table 2
Comparative plasma pharmacokinetic parameters (mean ± SD, n = 6 per group) for albendazole sulfoxide (ABZSO) and albendazole sulfone ($ABZSO_2$) in plasma, obtained after the oral administration of albendazole (ABZ) parent drug (5 mg/kg) either alone or co-administered with geraniol (GNL, two oral doses of 100 mg/kg, at -1 and 9 h post-administration of ABZ) to lambs.

Kinetic parameters	ABZSO		$ABZSO_2$	
	ABZ	GNL + ABZ	ABZ	GNL + ABZ
T ½ for. (h)	4.7 ± 1.31	5.08 ± 1.28	8.87 ± 2.67	8.64 ± 1.94
Cmax (µg/ml)	1.77 ± 0.46	1.97 ± 0.48	0.50 ± 0.13	0.52 ± 0.17
T max (h)	19.0 ± 7.01	22.0 ± 3.10	33.0 ± 9.10	34.0 ± 8.20
T ½ el. (h)	6.06 ± 2.77	7.38 ± 4.07	25.1 ± 17.9	29.9 ± 31.8
AUC_{0-t} (µg hr/ml)	50.5 ± 11.4	54.4 ± 10.4	18.1 ± 4.49	18.9 ± 5.08
MRT (h)	20.8 ± 1.76	22.1 ± 1.97	33.89 ± 3.41	34.6 ± 2.59

*T ½ for, metabolite formation half-life; Cmax, peak plasma concentration; Tmax, time to peak plasma concentration; T ½ el, elimination half-life; AUC_{0-t}, area under concentration vs. time curve from time 0 to the last concentration detected; MRT, mean residence time.
There was no significant difference between treatment means (P > 0.05)

4. Discussion

Although the main anthelmintic drugs such as benzimidazoles, imidazothiazoles and macrocyclic lactones were initially very effective, the intensive use in livestock led to resistance to all existing drug groups (Kaplan, 2020). The estimated time for resistance development from the launch of a new drug is less than 10 years (Kaplan, 2004) and the increasing reports of anthelmintic resistance in livestock have caused concern in recent years (Kaplan & Vidyashankar, 2012). In an attempt to manage anthelmintic resistance in ruminants, new compounds with anthelmintic activity or their combination with synthetic drugs are proposed as very promising tools (Lanusse et al., 2018). Phytotherapy offers a relevant field for exploring new therapeutic options with the challenge of reducing the use of synthetic drugs. In this context, natural compounds such as monoterpenes have the potential to be a source of new therapeutic agents (Nixon et al., 2020). GNL, a monoterpene found in several essential oils extracted from aromatic plants, has a wide range of pharmacological activities, including antimicrobial, antioxidant, neuroprotective, and anticancer properties (Pavan et al., 2018). Phytochemicals may alter the effectiveness of certain drugs by synergizing or antagonizing their therapeutic effects through drug-drug interactions at the plasma, cellular, or receptor level (Ondieki et al., 2017). This work characterized *in vitro* and *in vivo* the pharmacological features of GNL, evaluating for the first time its pharmacokinetic interaction with ABZ and, additionally, the *in vivo* efficacy of GNL against *H. contortus* in lambs.

Induction and/or inhibition of drug-metabolizing enzymes may affect the therapeutic efficacy and/or toxicity of co-administered drugs (Lanusse et al., 2018). The primary purpose of using enzyme inhibitors is to delay the metabolic conversion into a less potent or inactive metabolite while extending the presence of the more active parent drug. The role of FMO and CYP mixed-function oxidases in the hepatic S-oxygenation of ABZ in sheep has been thoroughly explored (Virkel et al., 2004; Velik et al., 2005). It has been shown that FMO-dependent metabolism is responsible for roughly 60–85% of ABZ hepatic S-oxidation (Miró et al., 2020c; Virkel et al., 2004; Virkel et al., 2014). Although to a lesser extent, one or more CYP isozymes are thought to participate in the sequential biotransformation of the anthelmintic, particularly the CYP1A subfamily in the sulfonation reaction (Benoit et al., 1992; Galtier et al., 1991; Velik et al., 2005). Several studies have attempted to increase ABZ systemic exposure and anthelmintic efficacy by inhibiting their FMO and/or CYP-dependent metabolism upon co-administration with other drugs (Lanusse & Prichard, 1993; Virkel et al., 2014).

As a result of evolution, plants produced chemicals that exert toxic effects on herbivorous predators, while animals developed specific enzymes as defensive mechanisms (Wöll et al., 2013). However, interactions of plant-derived chemicals with drug-metabolizing enzymes could be beneficial under certain circumstances. Within this context, plant secondary metabolites could be considered as pharmacological tools to increase the systemic exposure of active anthelmintic compounds. In fact, previous studies showed that the monoterpene thymol (TML) inhibited both the FMO- and the CYP1A1-dependent metabolism in sheep liver microsomes and delayed the S-oxygenation of ABZ by these pathways (Miró et al. 2020b; Miró et al., 2020c). Despite these

inhibitory effects observed *in vitro*, the monoterpene did not improve the systemic exposure of ABZ metabolites (Miró et al., 2020b). In the current trial, GNL was able to decrease the hepatic CYP1A1, CYP1A2 and FMO pathways of drug metabolism by roughly 41 to 91 % according to the enzyme activity tested (Fig. 1). Similarly, GNL caused a potent inhibition of CYP2B6-dependent metabolism in human liver microsomes (Seo et al., 2008). In addition to the inhibitory effect on CYP1A- and FMO-dependent activities, GNL affected the production of ABZSO and $ABZSO_2$ in sheep liver microsomes. These *in vitro* results indicate that GNL is a compound with the potential to be evaluated *in vivo* as an inhibitor of the metabolism of ABZ.

The rumen is a main organ of extra-hepatic biotransformation, particularly by reductive reactions (Renwick et al., 1986). The metabolite ABZSO is reduced to its ABZ parent drug and this metabolic reduction is critical to the antiparasitic efficacy of benzimidazoles in ruminants, since the parent drug has higher affinity to beta-tubulin (Lanusse et al., 1992). TML was recently found to completely abolish the formation of ABZ from ABZSO in ruminal incubations (Miró et al., 2020b) due to potent antimicrobial properties with a MIC against gram-positive and negative bacteria of 150 µg/mL (Nagoor Meeran et al., 2017). In that case, TML modified the ruminal environment, limiting ABZ availability in the gastrointestinal system and inducing a negative pharmacokinetic interaction between the monoterpene and ABZ (Miró et al., 2020b). In the present work, the presence of GNL did not affect the sulforeduction of ABZSO to the ABZ parent drug in rumen content (Table 1). The antibacterial activity of GNL appears to be weaker than that observed for TML. The MIC of GNL for most of the bacterium families tested is between 1300 and 5000 µg/mL (Lira et al., 2020), that is 9- – 33-fold higher than the MIC of TML and also several times greater than GNL concentrations used in the current study (0.52 and 5.19 mM, representing 80 and 800 µg/mL, respectively). Therefore, the co-administration of ABZ and GNL did not generate a negative *in vivo* metabolic interaction within the ruminal environment.

Based on the evidence that GNL delayed the formation of ABZ metabolites and also that GNL did not affect the ruminal sulforeduction of ABZSO, the next step was to analyze the *in vivo* phamarcokinetic interaction between ABZ and GNL in lambs. GNL was administered 1 h before and 9 h post-treatment of ABZ in an attempt to delay the ABZ metabolism during the main period of ABZSO formation (mean Tmax 19 h). Unlike expected, no changes in the systemic exposure of ABZSO or $ABZSO_2$ were observed after the administration of ABZ and GNL in combination (Fig. 3). Similar pharmacokinetic parameters for ABZSO and $ABZSO_2$ were observed in lambs after the coadministration of ABZ and GNL compared to the treatment with ABZ alone (Table 2). The indispensable condition for a metabolic interaction with clinical impact between two drugs is the simultaneous presence of sufficient concentrations of both molecules in the target site. In the present work, low amounts of GNL were detected in plasma in the current trial with a very short residence time in the systemic circulation. The mean concentration of GNL measured in plasma was 3.04 ± 1.33 µg/mL (representing 0.02 mM), only detected after 1 h post- second administration.

Only few studies have investigated the pharmacokinetics of this monoterpene and none in sheep. Pavan et al. (2018) administered an intravenous infusion of 12.5 mg of GNL to rats and observed a very short elimination half-life (about 12 min), which means that 99.9% of GNL was eliminated after 2 h. Besides, after the oral administration of GNL to rats, the Cmax in the bloodstream was achieved at 30 min post-treatment, which reflects the rapid absorption of GNL (Pavan et al., 2018). Another study shows that 90% of the monoterpene was hydrolyzed after 120 min of GNL incubation in rat liver homogenates, showing that the liver is particularly active in the metabolism of GNL (de Oliveira Junior et al., 2020). All these findings corroborate that GNL is rapidly removed from the body and that *in vivo* concentrations measured in lambs in the current trial were insufficient to prevent ABZ metabolism in the liver, unlike *in vitro* observations.

The main gastrointestinal nematodes affecting ruminants are located in abomasal and intestinal contents and mucosa. Any molecule with potential anthelmintic effect needs to reach effective concentrations at the target tissue for a certain period to assure their efficacy (Lifschitz et al., 2017). Considering the fast elimination observed after the administration of GNL formulated as an emulsion, GNL was encapsulated into yeasts in the present study with the aim of prolonging its permanence in the body and increasing the period of contact with the nematodes. Besides, GNL was administered during four days as a strategy to achieve drug accumulation in the target sites. GNL was detected in plasma only at 1 h post-administration of each daily dose. The mean plasma levels were between 0.71 ± 0.5 and 1.42 ± 2.06 µg/mL during the four days of treatment; these concentrations are even lower than those found in the pharmacokinetic assay after the emulsion administration (3.04 µg/mL). Under these experimental conditions, the FECR in lambs infected with *H. contortus* was 40.5% on day 14 post-administration. The encapsulation of carvacrol in yeast showed some advantages in the *in vitro* ectoparasiticide activity. The LC50 against *Rhipicephalus microplus* decreased 2.5-fold after the encapsulation of carvacrol in *Saccharomyces cerevisiae* compared to the non-encapsulated monoterpene (da Silva Lima et al., 2017). The effect of several encapsulated monoterpenes against *Ancylostoma ceylanicum* and *Trichuris muris* was recently evaluated *in vitro* (Mirza et al., 2020). GNL was classified as fast acting at a moderately high dose with a minimum effective dose between 200 and 333 µg/mL. Therefore, in our trial, the administration of encapsulated GNL showed an *in vivo* antiparasitic effect but lower than the threshold required for the synthetic molecules by regulatory agencies. Our experimental approach for this issue may show some limitations. Although the evaluation of the anthelmintic effect may be based on biomarkers of parasite infection such as FEC, this biomarker may not reflect the efficacy of GNL against the underlying parasite population. Besides, the evaluation of the FECR was carried out with a low number of animals. However these preliminary data may be useful for planning further trials and are in agreement with the few *in vivo* efficacy trials carried out with monoterpenes in ruminants. The long-term administration (45 days) of encapsulated carvone and anethole at 50 mg/kg to lambs infected with *H. contortus* reduced the eggs count approximately by 50% (Katiki et al., 2019). The single administration of essential oil of *Thymus vulgaris* or a combination of linalool/estragole, both at 100 mg/kg, to sheep naturally infected with gastrointestinal nematodes reduced the eggs count by 25% on day 14 post-administration (Štrbac et al., 2022). In this scenario, the potential clinical use of monoterpenes such as GNL seems promising but in an optimization context of proper dose regimen and pharmaceutical preparations. The future challenge for using these compounds as antiparasitic agents depends on the development of acceptable pharmaceutical formulations that provide sustained concentrations in the target tissues.

5. Conclusion

This study characterized the pharmacokinetic features of GNL in lambs, the drug-drug interactions with ABZ and its effect on FEC in lambs infected with *H. contortus* using *in vitro-in vivo* integrated experimental approaches. Knowledge of *in vivo* pharmacokinetics is essential to design appropriate bioactive formulations and adequate therapeutic protocols. Considering the increasing resistance of gastrointestinal nematodes to the different drug families, the role of different phytochemicals in the control of parasitic diseases deserves to be studied deeply for developing new pharmacological tools.

6. Ethics statement

Animal procedures and management protocols were carried out according to internationally accepted animal welfare guidelines (AVMA, 2007) and *Animal Welfare Policy (Academic* Council Resolution 087/02) approved by the Animal Welfare Committee of the *Faculty of* Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos

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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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References

Abdel-Rahman, F. H., Alaniz, N. M., & Saleh, M. A. (2013). Nematicidal activity of terpenoids. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes, 48*(1), 16–22. https://doi.org/10.1080/03601234.2012.716686

Alvarez, L., Lifschitz, A., Entrocasso, C., Manazza, J., Mottier, L., Borda, B., & Lanusse, C. (2008). Evaluation of the interaction between ivermectin and albendazole following their combined use in lambs. *Journal of Veterinary Pharmacology and Therapeutics, 31* (3), 230–239. https://doi.org/10.1111/j.1365-2885.2008.00953.x

Benoit, E., Besse, S., & Delatour, P. (1992). Effect of repeated doses of albendazole on enantiomerism of its sulfoxide metabolite in goats. *American Journal of Veterinary Research, 53*(9), 1663–1665.

Bishop, J. R., Nelson, G., & Lamb, J. (1998). Microencapsulation in yeast cells. *Journal of Microencapsulation, 15*(6), 761–773. https://doi.org/10.3109/02652049809008259

Capolongo, F., Santi, A., Anfossi, P., & Montesissa, C. (2010). Benzydamine as a useful substrate of hepatic flavin-containing monooxygenase activity in veterinary species. *Journal of Veterinary Pharmacology and Therapeutics, 33*(4), 341–346. https://doi.org/10.1111/j.1365-2885.2009.01145.x

Coles, G. C., Bauer, C., Borgsteede, F. H., Geerts, S., Klei, T. R., Taylor, M. A., & Waller, P. J. (1992). World association for the advancement of veterinary parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology, 44*(1-2), 35–44. https://doi.org/10.1016/0304-4017(92)90141-u

da Silva Lima, A., Pereira Maciel, A., Silva Mendonça, S., & Costa Junior, L. (2017). Use of encapsulated carvacrol with yeast cell walls to control resistant strains of Rhipicephalus microplus (Acari: Ixodidae). *Industrial Crops and Products, 108*, 190–194. https://doi.org/10.1016/j.indcrop.2017.06.037

de Oliveira Junior, E. R., Truzzi, E., Ferraro, L., Fogagnolo, M., Pavan, B., Beggiato, S., & Dalpiaz, A. (2020). Nasal administration of nanoencapsulated geraniol/ursodeoxycholic acid conjugate: Towards a new approach for the management of Parkinson's disease. *Journal of Controlled Release, 321*, 540–552. https://doi.org/10.1016/j.jconrel.2020.02.033

Galtier, P., Alvinerie, M., Plusquellec, Y., Tufenkji, A. E., & Houin, G. (1991). Decrease in albendazole sulphonation during experimental fascioliasis in sheep. *Xenobiotica; the Fate of foreign Compounds in Biological Systems, 21*(7), 917–924. https://doi.org/10.3109/00498259109039531

Galtier, P., Alvinerie, M., & Delatour, P. (1986). In vitro sulfoxidation of albendazole by ovine liver microsomes: Assay and frequency of various xenobiotics. *American Journal of Veterinary Research, 47*(2), 447–450.

Gibaldi, M., & Perrier, D. (1982). *Pharmacokinetics* (2nd edn, pp. 145–198). New York: Marcel Dekker, 1982.

Gilleard, J. S., Kotze, A. C., Leathwick, D., Nisbet, A. J., McNeilly, T. N., & Besier, B. (2021). A journey through 50 years of research relevant to the control of gastrointestinal nematodes in ruminant livestock and thoughts on future directions. *International Journal for Parasitology, 51*(13–14), 1133–1151. https://doi.org/10.1016/j.ijpara.2021.10.007

Helal, M. A., Abdel-Gawad, A. M., Kandil, O. M., Khalifa, M. M. E., Cave, G. W. V., Morrison, A. A., & Elsheikha, H. M. (2020). Nematocidal effects of a coriander essential oil and five pure principles on the infective larvae of major ovine gastrointestinal nematodes in vitro. *Pathogens, 9*(9), 1–17. https://doi.org/10.3390/pathogens9090740

Kaplan, R. M. (2020). Biology, epidemiology, diagnosis, and management of anthelmintic resistance in gastrointestinal nematodes of livestock. *Veterinary Clinics of North America - Food Animal Practice, 36*(1), 17–30. https://doi.org/10.1016/j.cvfa.2019.12.001

Kaplan, R. M., & Vidyashankar, A. N. (2012). An inconvenient truth: Global worming and anthelmintic resistance. *Veterinary Parasitology, 186*(1–2), 70–78. https://doi.org/10.1016/j.vetpar.2011.11.048

Kaplan, R. M. (2004). Drug resistance in nematodes of veterinary importance: A status report. *Trends in Parasitology, 20*(10), 477–481.

Katiki, L. M., Araujo, R. C., L., Gomes, A. C. P., Gutmanis, G., Rodrigues, L., Bueno, M. S., Veríssimo, C. J., Louvandini, H., Ferreira, J. F. S., & Amarante, A. F. T (2019). Evaluation of encapsulated anethole and carvone in lambs artificially and naturally-infected with *Haemonchus contortus*. *Experimental Parasitology, 197*, 36–42. https://doi.org/10.1016/j.exppara.2019.01.002

Lanusse, C., Canton, C., Virkel, G., Alvarez, L., Costa-Junior, L., & Lifschitz, A. (2018). Strategies to optimize the efficacy of anthelmintic drugs in ruminants. *Trends in Parasitology, 34*(8), 664–682. https://doi.org/10.1016/j.pt.2018.05.005

Lanusse, C. E., & Prichard, R. K. (1993). Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics. *Veterinary Parasitology, 49* (2–4), 123–158. https://doi.org/10.1016/0304-4017(93)90115-4

Lanusse, C. E., Nare, B., Gascon, L. H., & Prichard, R. K. (1992). Metabolism of albendazole and albendazole sulphoxide by ruminal and intestinal fluids of sheep and cattle. *Xenobiotica, 22*(4), 419–426. https://doi.org/10.3109/00498259209046653

Lapczynski, A., Bhatia, S. P., Foxenberg, R. J., Letizia, C. S., & Api, A. M. (2008). Fragrance material review on geraniol. *Food and Chemical Toxicology : An International Journal Published for the British Industrial Biological Research Association, 46*(1), S160–S170. https://doi.org/10.1016/j.fct.2008.06.048. Suppl.

Lifschitz, A., Lanusse, C., & Alvarez, L. (2017). Host pharmacokinetics and drug accumulation of anthelmintics within target helminth parasites of ruminants. *New Zealand Veterinary Journal, 65*(4), 176–184. https://doi.org/10.1080/00480169.2017.1317222

Lira, M. H. P., de, Andrade Júnior, F. P.de, Moraes, G. F. Q., Macena, G., da, S., Pereira, F., de, O., & Lima, I. O. (2020). Antimicrobial activity of geraniol: An integrative review. *Journal of Essential Oil Research, 32*(3), 187–197. https://doi.org/10.1080/10412905.2020.1745697

Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Maté, L., Virkel, G., Lifschitz, A., Ballent, M., & Lanusse, C. (2008). Hepatic and extra-hepatic metabolic pathways involved in flubendazole biotransformation in sheep. *Biochemical Pharmacology, 76*(6), 773–783. https://doi.org/10.1016/j.bcp.2008.07.002

Maté, L, Ballent, M, Cantón, C, Ceballos, L, Lifschitz, A, Lanusse, C, Alvarez, L, & Liron, JP (2018). Assessment of P-glycoprotein gene expression in adult stage of Haemonchus contortus in vivo exposed to ivermectin. *Veterinary parasitology, 264*, 1–7. https://doi.org/10.1016/j.vetpar.2018.10.011

McKenna, P. B. (1990). The detection of anthelmintic resistance by the faecal egg count reduction test: an examination of some of the factors affecting performance and interpretation. *New Zealand Veterinary Journal, 38*(4), 142–147.

Miró, M. V., Luque, S., Cardozo, P., Lloberas, M., Sousa, D. M., Soares, A. M. S., Costa-Junior, L. M., Virkel, G. L., & Lifschitz, A. L. (2020a). Plant-derived compounds as a tool for the control of gastrointestinal nematodes: Modulation of abamectin pharmacological action by carvone. *Frontiers in Veterinary Science, 7*, 1–10. https://doi.org/10.3389/fvets.2020.601750

Miró, M. V., Silva, C. R., Viviani, P., Luque, S., Lloberas, M., Costa-Júnior, L. M., Lanusse, C., Virkel, G., & Lifschitz, A. (2020b). Combination of bioactive phytochemicals and synthetic anthelmintics: In vivo and in vitro assessment of the albendazole-thymol association. *Veterinary Parasitology, 281*. https://doi.org/10.1016/j.vetpar.2020.109121. Article 109121.

Miró, V., Lifschitz, A., Viviani, P., Rocha, C., Lanusse, C., Costa, L., & Virkel, G. (2020c). *In vitro* inhibition of the hepatic S-oxygenation of the anthelmintic albendazole by the natural monoterpene thymol in sheep. *Xenobiotica, 50*(4), 408–414. https://doi.org/10.1080/00498254.2019.1644390

Mirza, Z., Soto, E. R., Hu, Y., Nguyen, T. T., Koch, D., Aroian, R. V., & Ostroff, G. R. (2020). Anthelmintic activity of yeast particle-encapsulated terpenes. *Molecules, 25* (13). https://doi.org/10.3390/molecules25132958. Article 2958.

Nagoor Meeran, M. F., Javed, H., Al Taee, H., Azimullah, S., & Ojha, S. K. (2017). Pharmacological properties and molecular mechanisms of thymol: Prospects for its therapeutic potential and pharmaceutical development. *Frontiers in Pharmacology, 8*. https://doi.org/10.3389/fphar.2017.00380. Article 380.

Nelson, G., Duckham, S. C., & Crothers, M. E. D. (2006). Microencapsulation in yeast cells and applications in drug delivery. *Polymeric Drug Delivery I. Chapter, 19*, 268–281.

Nixon, S. A., Welz, C., Woods, D. J., Costa-Junior, L., Zamanian, M., & Martin, R. J. (2020). Where are all the anthelmintics? Challenges and opportunities on the path to new anthelmintics. *International Journal for Parasitology: Drugs and Drug Resistance, 14*, 8–16. https://doi.org/10.1016/j.ijpddr.2020.07.001

Ondieki, G., Nyagblordzro, M., Kikete, S., Liang, R., Wang, L., & He, X. (2017). Cytochrome P450 and P-glycoprotein-mediated interactions involving African herbs indicated for common noncommunicable diseases. *Evidence-Based Complementary and Alternative Medicine, 2017*. https://doi.org/10.1155/2017/2582463. Article 2582463.

Pavan, B., Dalpiaz, A., Marani, L., Beggiato, S., Ferraro, L., Canistro, D., & Spisni, E. (2018). Geraniol pharmacokinetics, bioavailability and its multiple effects on the liver antioxidant and xenobiotic-metabolizing enzymes. *Frontiers in Pharmacology, 9*, 18. https://doi.org/10.3389/fphar.2018.00018

Pegolo, S., Merlanti, R., Giantin, M., Dacasto, M., Montesissa, C., & Capolongo, F. (2010). High performance liquid chromatography determination of cytochrome P450 1A and

2C activities in bovine liver microsomes. *Veterinary Journal, 183*(1), 81–88. https://doi.org/10.1016/j.tvjl.2008.08.006

Peña-Espinoza, M., Valente, A., Thamsborg, S. M., Simonsen, H. T., Boas, U., Enemark, H. L., & Williams, A. (2018). Anti-parasitic activity of chicory (Cichorium intybus) and the role of its natural bioactive compounds : A review. *Parasites and Vectors, 11*(1), 1–14.

Renwick, A. G., Strong, H. A., & George, C. F. (1986). The role of the gut flora in the reduction of sulphoxide containing drugs. *Biochemical Pharmacology, 35*(1), 64. https://doi.org/10.1016/0006-2952(86)90557-5. –64.

Roberts, F. H. S., & O'sullivan, P. J. (1950). Methods for egg counts and larval cultures for strongyles infesting the gastro-intestinal tract of cattle. *Australian Journal of Agricultural Research, 1*(1), 99–102.

Seo, K. A., Kim, H., Ku, H. Y., Ahn, H. J., Park, S. J., Bae, S. K., & Liu, K. H. (2008). The monoterpenoids citral and geraniol are moderate inhibitors of CYP2B6 hydroxylase activity. *Chemico-Biological Interactions, 174*(3), 141–146. https://doi.org/10.1016/j.cbi.2008.06.003

Štrbac, F., Bosco, A., Maurelli, M. P., Ratajac, R., Stojanović, D., Simin, N., Orčić, D., Pušić, I., Krnjajić, S., Sotiraki, S., Saralli, G., Cringoli, G., & Rinaldi, L. (2022). Anthelmintic properties of essential oils to control gastrointestinal nematodes in sheep—In vitro and in vivo studies. *Veterinary Science, 9*. https://doi.org/10.3390/vetsci9020093. Article 93.

Velik, J., Szotakova, B., Baliharova, V., Lamka, J., Šavlík, M., Wsol, V., & Skalova, L. (2005). Albendazole repeated administration induces cytochromes P4501A and accelerates albendazole deactivation in mouflon (Ovis musimon). *Research in Veterinary Science, 78*(3), 255–263.

Virkel, G., Lifschitz, A., Sallovitz, J., Maté, L., Farías, C., & Lanusse, C. (2014). In vitro and in vivo assessment of the benzydamine-mediated interference with the hepatic S-oxidation of the anthelmintic albendazole in sheep. *Small Ruminant Research, 120*(1), 142–149. https://doi.org/10.1016/j.smallrumres.2014.04.009

Virkel, G., Lifschitz, A., Sallovitz, J., Pis, A., & Lanusse, C. (2004). Comparative hepatic and extrahepatic enantioselective sulfoxidation of albendazole and fenbendazole in sheep and cattle. *Drug Metabolism and Disposition, 32*(5), 536–544. https://doi.org/10.1124/dmd.32.5.536

Virkel, G., Lifschitz, A., Pis, A., & Lanusse, C. (2002). In vitro ruminal biotransformation of benzimidazole sulphoxide anthelmintics: Enantioselective sulphoreduction in sheep and cattle. *Journal of Veterinary Pharmacology and Therapeutics, 25*(1), 15–23. https://doi.org/10.1046/j.1365-2885.2002.00373.x

Wöll, S., Kim, S. H., Greten, H. J., & Efferth, T. (2013). Animal plant warfare and secondary metabolite evolution. *Natural Products and Bioprospecting, 3*(1), 1–7. https://doi.org/10.1007/s13659-013-0004-0