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Anthelmintic evaluation of selected phytochemicals in dairy ewes

dr. Elisa Ortu

Direttore della Scuola	prof. Alba Pusino
Referente di Indirizzo	prof. Nicolò P.P. Macciotta
Docente Guida	dr. Gianni Battacone
Tutor Guida	dr. Pierluigi Caboni

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APPENDIX

List of Abbreviations

ABZ: albendazole

ABZ-SO: albendazole sulfoxide

ABZ-SO2: albendazole sulfone

ADME: absorption, distribution, metabolism and elimination

ALB: albumin

ALP: alkaline phosphatise

ASH: ashes

ATP: adenosine triphosphate

AWE: *A. altissima* wood extract

BASOS: basophils cells

BCS: body condition score

BZD: benzimidazoles

BT: total bilirubine

BW: body weight

CBC: complete blood count

CH: haemoglobin content

CP: crude protein

CRE: creatinine

DAD: diode array detector

2,4-DEC: (E,E)-2-4-decadienal

DM: dry matter

EC_{50}: half maximal effective concentration

ECG: epigallocatechin gallate

EDTA: ethylenediaminetetraacetic acid

EOS: eosinophils Cells

EPG: eggs per gram of feces

ESI: electrospray ionization

F: furfural

FA: 2-furoic acid

FECRT: fecal egg count reduction test

FID: flame ionization detector

FWECs: faecal worm egg counts

g: gram

g/d: gram per day

GABA: gamma amino butyric acid

GC-MS: gas chromatography- tandem mass spectrometry

GGT: gamma glutamil transpeptidase

GI: gastrointestinal

GIN: gastrointestinal nematode

GOT: glutamic oxaloacetic transaminase

GPT: glutamic pyruvic transaminase

h: hours

2-HBA: 2-hydroxybenzaldehyde

HCT: hematocrit

HDW: haemoglobin distribution width

HGB: hemoglobin

5-HMF: 5-Hydroxymethylfurfural

HPLC: high performance liquid chromatography

kg: kilogram

L_1: first stage larvae

L_2: second stage larvae

L_3: third stage larvae

L_4: fourth stage larvae

LC_{50}:lethal concentration 50

LC-MS: liquid chromatography tandem mass spectrometry

LC-QTOF/MS: liquid chromatography tandem Mass Quadrupole Time-of Flight

LD_{50}: lethal dose 50

LEVAMISOLE HCl: levamisole hydrochloride

LIMPHS: lymphocytes cells

LOD: detection limit

LOQ: quantification limit

LUC: large unstained cells

M:molarity

MCH: mean corpuscular haemoglobin

MCHC: mean corpuscular hemoglobin concentration

MCV: mean corpuscular volume of red blood cells

mg: milligram

$MgSO_4$: magnesium sulphate

mL: millilitre

mM: millimolar

MONOS: monocytes cells

MPV: mean platelet volume

N: normal

NDF: neutral detergent fiber

NEUTS: neutrophils cells

PBS: phosphate-buffered saline solution

PCR: polymerase chain reaction

PLT: total platelets

PROT: total protein

RBC: red blood cells

RDW: cells distribution width

REO: *R. Chalepensis* essential oil

RME: *R. Chalepensis* methanol extract

RPM: rotation for minute

SA: salicylic acid

SCC: somatic cell count

SD: standard deviation

S/N: signal noise ratio

TCA: trichloroacetic acid

2-UND: 2-undecanone

UV: ultraviolet

WBCB: white blood cells

General abstract

In this dissertation we showed the *in vitro* and *in vivo* anthelmintic activity of selected plant extracts and secondary metabolites against gastrointestinal nematodes and their effects on Sarda dairy ewes. The first chapter reviewed the effects of gastrointestinal nematodes and lungworms on the health status and welfare of small ruminants and the strategies to control nematode infestations. Moreover, the anthelmintic properties of the plant extracts are illustrated. The second chapter reported the *in vitro* anthelmintic activity of *Ruta chalepensis* L. extracts and of selected phytochemicals against gastrointestinal nematodes and lungworms of sheep. Especially, *Ruta chalepensis* L. methanol extract and 2-undecanone showed the highest nematicidal activity with an $EC_{50/96h}$= 0.10 mg/mL and $EC_{50/24h}$= 0.88 mg/mL, respectively. The data revealed *in vitro* dose-dependent anthelmintic activity. In the third chapter we reported the experiment where increasing doses of furfural and 2-hydroxybenzaldehyde were orally administered to Sarda dairy ewes. These phytochemicals and their metabolites were not detected in milk, urine and faecal samples. Moreover, the ewe blood parameters were within the normal range. In the fourth chapter we reported that a single oral dose of furfural, 2-hydroxybenzaldehyde, 2-undecanone and albendazole was administered in sheep. The compounds and their metabolites were not detected in milk with the exception of albendazole sulfoxide a known metabolite of albendazole. Therefore, no negative effects were observed in animals treated with these compounds. With regard to anthelmintic activity, the furfural, 2-hydroxybenzaldehyde, 2-undecanone groups showed the lowest activity if compared with albendazole. In the future, we will optimise the dosage and the mode of action of furfural, 2-hydroxybenzaldehyde and 2-undecanone.

CHAPTER 1

Effects of gastrointestinal nematodes and lungworms on the health status and welfare of small ruminants. Strategies to control nematode infestations

Introduction

Elisa Ortu

1. Introduction

Infections by gastrointestinal nematodes (GIN) is one of the main global issue that involve small ruminant herds. They are responsible in sheep and goats to important economic losses such as reduction of milk yield, growth and body weight. It is difficult evaluate the annual cost of the anthelmintic infections because depends on climate conditions, geographical regions and nematode species (Sutherland and Scott, 2010). Goats are more susceptible to gastrointestinal (GI) parasites than sheep because in goats the acquisition of immune responses is less efficient (Zanzani et al., 2014). GIN reduce the animal health status and welfare, limit productivity of sensitive animals because nematodes remove nutrients from the ingesta and they have indirect effects on immune response (Sargison, 2012). Especially, *Haemonchus contortus*, one of the most pathogenic parasite in small ruminants is capable to cause acute disease feeding on blood and producing anemia in the host. GI parasites in goats have consequences on patho-physiology causing an increasing on blood eosinophils and a reduction on milk production but on the whole these effects do not reduce fat and protein content (Hoste and Chartier, 1998; Veneziano et al., 2004). Small ruminants are very susceptible to helminthiasis and nematodes can compromise the health status of the host causing in several cases the death of the animal. Global small ruminant farming are largely influenced by the conversion of nutrients to meat and milk; control of GIN is essential in compliance with the economic requirements (Sargison, 2011). Most infections remain at the subclinical level and can promote the decrease of milk and meat production. The control of this problem is quite critical in sheep and goats for the development of resistance of these parasites against conventional chemical anthelmintic drugs. The anthelmintics used against GIN are employed also to control lungworm infestations; these nematodes are responsible to pathogenic effects such as bronchitis, pneumonia or in several cases fatal outcomes depending on nematode type and on number of larvae ingested. Therefore, pulmonary worms in small ruminants as wells as GIN, are responsible to decreasing of productivity and mortality. Helminthiasis control in sheep and goats is essential to animal health and to limit negative effects on production of food use such as minimize the withdrawal period on milk and meat for human use after drug treatments of the animals. To realize this control is basic realize management changes and apply a multiple strategy including accurate monitoring of

parasite infestations, grazing management methods to minimize the contact between the host and the infective stage larvae, genetic selection to reduce anthelmintic resistance, vaccination, selected feed supplementation to improve host resistance or resilience against nematodes, proper and tactical use of chemical anthelmintic drugs. Moreover, in the last twenty years the use of forages and plants with anthelmintic properties is growing and for this reason they are considered a valid alternative to conventional anthelmintic drugs (Torres-Acosta and Hoste, 2008; Hoste and Torres-Acosta, 2011).

2. Life cycle of nematodes in small ruminants

The GIN in small ruminants are located in different sites depending on development stage and nematode species. Generally, adult nematodes of *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus axei* reside in the abomasum, while *Bunostomum trigonocephalum*, *Cooperia curticei, Nematodirus* spp., *Trichostrongylus* spp*., Oesophagostomum columbianum* and *Chabertia ovina* are located in small and large intestine (Makovcová et al., 2008). The life cycle of GIN starts when small ruminants ingest through the pasture the infective third stage larvae (L_3) and the immature worm become adult in the abomasum or intestine of the host. Adult female worms can produce up to 10,000 eggs per day which are excreted in the soil through host feces and takes place a hatching of the eggs to first stage larvae (L_1). L_1 are immature worms and their development to infective L_3 occurs through two moults on pasture and depends on temperature and moisture levels. During the moults, larvae feed bacteria that find into pasture; ruminants are infected by the ingestion of the infective L_3 that posses a protective cuticle and for this reason is less susceptible to hostile climate conditions. When ruminant ingests L_3, larvae travel to the gastrointestinal tract, where develop the fourth stage larvae (L_4) and become adults. Generally, after 21 days eggs produced to female worms appear in the feces and the life cycle start anew (Southwell et al., 2008). Usually, in the same animal there is a condition of poliparasitism that involve different species of GIN; monospecific infections are extremely rare. Pathogenic factors of single species can add through them and the result is a complication of the health status of the animal infected (Roeber et al., 2013; Scala, 2006). On the other hand, the life cycle of lungworms in small ruminants can be ranked

into two categories: direct and indirect. The life cycle of *Dictyocaulus filaria* that belong to Dictyocaulidae family is a direct cycle: sheep and goats ingest infective L_3 through the pasture and L_4 reach the lungs; after 3 or 4 weeks appear in the bronchi and trachea young adults (L_5) or mature adults depending on species. Adult female worms produce eggs which hatch in the small intestine after oviposition in the airways. L_1 pass in the pasture through host feces where develop the L_2 and later the L_3. Instead, life cycle of Protostrongylidae family which includes *Muellerius capillaris, Neostrongilus linearis*, *Cystocaulus ocreatus* and Protostrongylus spp. species is an indirect cycle because requires an intermediate host to develop the L_3. Adults females in the lungs produce eggs that hatch and move by way to intestine; L_1 pass in the pasture through feces where there is a development to L_2. The development of the infective L_3 depends on presence of gastropods such as snails and slugs; in absence of these intermediate hosts the worm is enable to complete its life cycle. Sheep and goats ingest mollusks that contain L_3 and in the lungs come the development to L_4, L_5 and later to adult worm (Panuska, 2006). GIN and lungworms cycles are influenced by the capacities to infective stage larvae to persist on pasture in extreme climate conditions or within the host when temperature and moisture conditions are unsuitable (Sutherland and Scott, 2010).

3. Helminthiasis symptoms

The severity of the disease produced by GI worm infections depends on the number and species of larvae ingested and is influenced by the age and health status of the host. Common signs of GIN infections are anorexia, diarrhea, weight loss, dental problem such as jaw lesions, arthritis and in the case of blood-feeding species such as *H. contortus*, include anemia and edema (Taylor et al., 2007). Each parasite with hematophagous action is able to remove by host about 0.05 mL of blood for day causing a weakening of breeding performance. Moreover, parasitic gastroenteritis produce in sheep and goats reduction of appetite, protein deficiency with modification in energy balance, malabsorption and maldigestion, imbalance of fluids and macroelements, and reduction of immune defenses (Hoste and Torres-Acosta, 2011; Sargison, 2011; Sargison, 2012). In addition, parasites cause tissue damages, promoting the invasion of

bacteria that remove energy and protein to body host. The combination of these physio-pathological effects reduce the production efficiency of small ruminants. Traditionally, GIN infestations are controlled using chemical anthelmintic drugs: the aim of these treatments is reduce the number of worms in the host to avoid clinical signs and safeguard welfare animal. Moreover, to ensure satisfactory animal production and to preserve the efficacy of the drugs that so far do not develop resistance problems (Sargison, 2008). On the other hand, lungworms in small ruminants are responsible to respiratory diseases. Especially, *D. filaria* is more pathogenic than Protostrongylidae family but the death is uncommon. Clinical signs include edema, collapse of lung tissue, loss of appetite, reduction of body weight, bacterial pneumonia and bronchitis. Sheep and goats develop immunity to reinfection and young animals are more susceptible to infections. Generally, infections induced by *M. capillaris* are not associated with clinical diseases, but in some cases are reported clinical signs such as pneumonia, lowering of pulmonary gas exchange, reduction on body weight and breeding performance (Panuska, 2006).

4. Pharmaceutical control of nematode parasites

Pharmaceutical drugs are essential to control GIN and lungworm infestations in association with other strategies. In small ruminant herds anthelmintic treatments increase milk production respect to animal untreated (Veneziano et al., 2004; Sechi et al. 2010). Before the introduction of these conventional drugs with broad spectrum of action, control of parasites occurred through the use of various crude medicines such as copper sulfate, sodium arsenite, carbon tetrachloride, tetrachloroethylene and nicotine sulfate. Frequently, these drugs showed the same toxicity both to nematodes and small ruminant hosts. In 1940s was introduced phenothiazine to control nematode infestations, but major progress was made in 1960s with the discovery of thiabendazole, following by levamisole in 1970s and ivermectin in 1981s. In 2010s was introduced monepantel, considered a new paradigm for the control of nematodes for its action, good tolerability and low toxicity (Kaminsky et al., 2008). Initially, ivermectin and benzimidazoles showed excellent broad spectrum activity against small ruminant nematodes and good safety for the host; later many studies of resistance were reported

by different authors caused by an intensive and uncontrolled use (McKellar and Jackson, 2004; Sargison, 2011). The efficacy of the anthelmintic drugs depends on the gravity of infestation and on the health status of the animal. Moreover, the effectiveness of a drug dependes on concentration used and on time that spends in its site of action. An anthelmintic drug should have the following peculiarities to be considered ideal:

- low toxicity: is basic to preserve health status and welfare of the host;
- efficacy against many nematode species at the same time: in condition of poliparasitism is an essential parameter to control the evolution of infestation;
- rapid metabolism and excretion: it is very important to excrete the drug quickly to reduce the withdrawal times of milk and meat for human consumption;
- pharmaceutical dosage form: this selection influences the complexity of administration and the absorption time of the drug. The use of excipients can help to improve the pharmacokinetic of the drug;
- down market: it is essential especially for zootechnical profits.

Drugs used nowadays can be ranked into six groups based on similar chemical structure and mode of action:

- benzimidazole derivatives (BZD): the first introduced was thiabendazole, followed by fenbendazole, albendazole, mebendazole and oxfendazole; they have a broad-spectrum of action. Their anthelmintic activity is due by degenerative alterations in the intestinal cells of the worm by selective binding to colchicine receptors of β-tubulin, thus inhibiting its polymerization and assembly into microtubules. The lossof the cytoplasmic microtubules reduces the uptake of glucose and glycogen stores with cellular degenerative changes and decreasing of adenosine triphosphate (ATP). ATP is essential for the production of energy required for the survival of the nematodes. The duration of the exposure of nematode to drug is the key of the efficacy of this group; for this reason many studies were made to extend this exposure time through different pharmaceutical dosage forms such as intraruminal boluses or association of drugs with similar chemical structure and mode of action. Depending on active ingredient used, the benzimidazole derivatives are used in cattle, sheep, goats, horses and pigs;

- imidazothiazole derivatives: levamisole and tetramisole act as a cholinergic agonist of nicotinic receptors in neuromuscular junctions of the nematodes, opening and blocking acetylcoline receptors with the result of worm paralysis. Morover, they show an interesting activity against lungworms (Thienpont et al., 1966; McKellar and Jackson, 2004). Depending on active ingredients, imidazothiazole derivatives are used in cattle, sheep, goats and pigs;

- macrocyclic lactones: include avermectins and milbemycins. They are generated naturally as fermentation products by soil actinomycetes *(Streptomyces* spp.) and some drugs are chemically modified. They interfere with the neurotransmission in nematodes inducing the release of gamma amino butyric acid (GABA). The substitution of the C-5 hydroxy with an oxo-substituent on ivermectin or doramectin reduce anthelmintic selective activity against *H. contortus* (Riviere and Papich, 2009). They show activity against GIN and lungworms and depending on country are used in cattle, sheep and goats (European Agency for the Evaluation of Medicinal Products);

- salicylanalide derivatives: the mode of action of closantel is complex but it can be considered as an uncoupler of the oxidative phosphorylation in the mitochondria cells of the nematode, which disturbs the production of ATP through suppression of the activity of succinate dehydrogenase and fumarate reductase. It is highly effective against GIN and especially against *H. contortus*; for this reason is used as a valid alternative to nematode resistance of ivermectin, levamisole, morantel and benzimidazole derivatives. In ruminants, closantel is well absorbed after enteral and parenteral administration;

- tetrahydropyrimidines derivatives: morantel is a selective nicotinic agonist of the synaptic and extrasynaptic acetylcholine receptors present in the neuromuscular junction of the nematodes. It is 100 times more potent than acetylcholine; for this reason mimes an excessive amount of the natural neurotransmitter producing contraction and spastic muscle paralysis in nematodes. In ruminants, morantel absorption is low and after oral administration in goats is not detectable in bloodstream; it is largely excreted unmodified through feces compartment (Riviere and Papich, 2009);

- amino-acetonitrile derivatives: monepantel acts on specific nicotinic acetylcholine receptor sub-unit Hco-MPTL-1 present in neuromuscular junctions of the worms. This binding acetylcholine-receptor is responsible for the correct transmission of the nerve signals; monepantel blocks these receptors generating paralysis in the worms. Monepantel sulfone is the main active metabolite and it is present in sheep bloodstream after intravenous and oral administration (Karadzovska et .al., 2009). Excretion of this drug is mainly via the feces within 3 days and a small amount in the urine after repeated administration. This drug is used in sheep to control GIN infestations but not against lungworms (European Medicines Agency, 2012). In 2012 the use was extended to goats; pharmacokinetic studies demonstrated that the elimination of monepantel sulfone is faster in goats than in sheep (European Agency for the Evaluation of Medicinal Products, 2012).

In the selection of an anthelmintic drug contribute also its absorption, distribution, metabolism and elimination (ADME). It is mandatory to control drug residues in sheep and goat products to assess food safety (Hoste, Torres- Acosta, 2011). Therefore, the drug should be not persistent in the edible tissues of food producing by animals (Riviere and Papich, 2009). Traces of drugs can be also found in milk and meat produced of small ruminants treated. Different withdrawal periods for milk and meat used for human consumption are mandatory in function of the anthelmintic drug administered. In monepantel (Zolvix formulation) the withdrawal period is 7 days for meat and offal in sheep and goats treated. Monepantel can be used in breeding sheep including pregnant and lactating ewes but not in female sheep producing milk for human consumption (EMA, 2012). For ivermectin (Ivomec formulation) the withdrawal period in cattle corresponds to 49 days for meat and offal when ivermectin is used as sole active ingredient or in combination with closantel. Ivermectin can not used in animal producing milk for human consumption (Annex I). On the other hand, for thiabendazole (Tibigel formulation) the withdrawal period corresponds to 35 days for meat and 11 days for milk in goats treated. Instead, for fenbendazole (Panacur formulation) the withdrawal period in sheep and goats corresponds to 28 for meat and 9 days for milk. In levamisole chlorohydrate (Citarin and Pamizole-L formulation) the withdrawal period in sheep and goats corresponds to 14 days for meat, whereas for morantel is 42 days for meat in goats. For albendazole (Valbazen formulation) the withdrawal period

corresponds to 4 days for meat, offal, and milk in sheep (Ministero della Salute, 7 Giugno 2012). Finally, in Febantel (Rintal formulation) the withdrawal period in sheep corresponds to 28 days for meat and 7.5 days for milk (European Agency for the Evaluation of Medicinal Products; D.M. 4 Marzo 2005).

5. Anthelmintic resistance

For a long time the control of GI parasites in small ruminants has been reached by the use of chemical anthelmintic drugs but nowadays is becoming more difficult control them by reason of development of resistance of these parasites to conventional drugs. The intensive use of anthelmintics for many years to control GI parasitism in small ruminants has led to the development of drug resistance to one or more classes of anthelmintics at the same times. Therefore, anthelmintic resistance is one of the most serious problem over the world associated to GIN that threaten sheep and goat herds both in temperate and tropical areas (Torres- Acosta and Hoste, 2008). In grazing conditions, goats are usually more infected than sheep and as a result develop resistance more quickly. This effect can be explain to their lower ability to develop immune responses against worms (Torres-Acosta et al., 2012a; Zanzani et al., 2014). Actually, in Europe GIN resistance is extremely variable, probably because many factors contribute to its development. Zanzani et al. (2014) determined the presence of anthelmintic resistance in 30% of the flocks of dairy goats studied in Northern Italy: benzimidazoles and probenzimidazoles appeared to create more resistance problems respect to macrocyclic lactones. In addition, the authors of this study submitted farmers to questionnaires with the objective of analyze the ordinary practices applied to control GIN. Through this instrument, authors realized the importance to educate farmers to an accountably use of correct doses of anthelmintic drugs and to take into consideration the possibility of a multi-strategy including a rotation of different classes of drugs (Zanzani et al., 2014). The problem of benzimidazoles resistance is reached also by Chartier (1998) that studied sheep and goat herds in west of France. Chartier found that the resistance to benzimidazoles was present in all 15 goat farms studied while in sheep the 83% of farms analyzed showed GIN resistant for benzimidazoles, especially the 50% of farms for levamisole, a drug belonging to imidazothiazole derivatives. Moreover, the

study indicated *Teladorsagia* spp., Trichostrongylus spp. and Cooperia spp.as the predominant resistant genera. Probably, the high frequency of GIN resistance to benzimidazole derivatives may be associated with an intensive and an under-dosage of these drugs (Chartier et al., 1998). Furthermore, Sargison (2011) affirmed that in the last years *T. circumcincta* and *H. contortus* have shown resistance to benzimidazole, imidazothiazole and macrocyclic lactone derivatives. On the other hand, in Norway the problem of resistance is less severe than in other countries of Europe: the efficacy of macrocyclic lactones was 100% in all sheep and goat farms monitored by Domke et al. (2012). On the other hand, the flocks classified as resistant for albendazole were 10.5% and 31.0%, respectively in the randomly and non-randomly sheep farms studied. In Guadeloupe GIN infections are responsible of the decreasing productivity of Creole goats raised for meat. In South America countries (Argentina, Brazil, Uruguay and Paraguay) is common to find multiple resistance in sheep farm as well as in the south of the United States of America, parts of Mexico and Costa Rica. In contrast, other American countries show low anthelmintic resistance problems (Torres-Acosta et al., 2012a). Anthelmintic resistance is influenced by different factors such as the drug efficacy, the frequency and dose of anthelmintic treatments, the species and the number of nematodes involved (Sargison, 2008). It is clear that in the last years the phenomenon of multi-drug resistance of GIN against benzimidazole, imidothiazole, and macrocyclic lactone derivatives is growing. For this reason, it is urgent to study and apply valid alternatives to use the of conventional anthelmintics such as operating in grazing management, using plants with anthelmintic properties, employing drugs with different mode of action respect to the conventional drugs.

6. Multi-strategies to control nematode infestations

A rapid and a traditional method used to monitor GIN infestations in ruminants is the fecal worm egg counts (FWECs). The specific identification of the L_3 species obtained by coprocolture is done using an optic microscope. On the other hand, the Baermann technique is used for the identification of the lungworm infections. Moreover, the fecal egg count reduction test (FECRT) is a routine method used to detect anthelmintic resistance: it use an easy and cheap procedure but the limit is that the resistant nematode

species is not clear. Furthermore, different *in vitro* technique are used to monitor worm resistance, such as the egg hatch assay, the larval development assay and the larval migration test. Individual analysis of the animals using the FWECs give more information but for economical reason pooled samples are sufficient (Coles et al., 2006; Sargison, 2008). Therefore, indirect markers to monitor parasitic diseases in the host can be used such as FARMACHA test (is based on the monitoring of conjunctival colour, as indicator of presence of anemia), body condition score (BCS), body weight and milk production trend. Moreover, serum pepsinogen level is considered an important parameter to monitor GIN infections causing by nematode with hematophagous action. Nowadays, polymerase chain reaction (PCR) is a modern molecular technologie that permit the identification of specific parasites and to obtain results with high sensitivity. This method represents an important option to resolve diagnostic problems relating to parasites. It would be useful to introduce routine molecular diagnostic techniques for the monitoring of parasite infestations and anthelmintic resistance with the aim to obtain results more specifics than the traditional method used until now (Gasser, 2006). GIN infestation in Guadeloupe is at fault of decreasing productivity of Creole goats raised for meat. The study showed that a genetic improvement could be a possibility of increase viability of goats kids during helminthiasis (Mandonnetet al., 2003). Strategic anthelmintic treatments include pharmacological treatment, rotation of grazing and the introduction of alternative plants with anthelmintic properties. For most small ruminant producers the control of nematode infestations is basic for their future prosperity; it is essential and urgent that the farmers understanding the seriousness of the problem. Frequently, in Lombardy (Italy) anthelmintic treatment in goat herds is realized inconstantly once or two for a year without parasitological analysis (Zanzani, 2014). Farmers should be educated to apply an effective nematode control and to reduce economic costs associated to useless treatments. A good strategy to control nematode infestation is carry out with an appropriate flock management. Individual sheep and goats differ in their tolerance to nematode infestation; for this reason a regular treatment only for the animals positive to nematode infestations would be useful to reduce the problem. Certainly, a targeted selective treatment is more complicated than routine treatments, because can be expensive and complicate to monitor each animal but this approach reduces the number of animals treated and consequently delay the development of resistance (Sargison,

2008). It is essential to reduce the contact between susceptible hosts and infective larvae in the pasture through grazing management methods. Moreover, the host defense capacities against GIN can be optimized through genetic selection and nutritional manipulation of small ruminants' diet (Hoste and Torres-Acosta, 2011; Torres-Acosta et al., 2012b). Concerning to the control of lungworm infestations, a strategic solution include the administration of conventional drugs to carrier animal to prevent contamination of pasture. The use of distinct grazing for susceptible animals respecting to infected animals and the implementation of dry pastures reduce the development and survival of larvae and the transmission of lungworm infestations. Furthermore, the reduction of contact through small ruminants and intermediate hosts can limit the chance of infection caused by Protostrongylidae family (Panuska, 2006). It is clear that anthelmintic drugs should be used as part of an integrated infestation control program, combined use of several tools such as resistance testing, worm and eggs monitoring, grazing management, avoid unnecessary treatments, apply annual drench rotation, optimize genetic selection and test nutritional manipulation (Sargison, 2008; Sutherland and Scott, 2010; Hoste and Torres-Acosta, 2011). In the last years, many plants with anthelmintic properties are studied. Their ability against nematodes depends on presence of one or more secondary metabolites that possess negative effects on parasites vitality. Nowadays, the condensed tannins are the most studied compounds with nematicidal activity in small ruminants infected by GIN (Hoste and Torres-Acosta, 2011; Torres-Acosta et al., 2012b).

7. Anthelmintic properties of plant extracts and *in vitro* studies

In the last years, the interest to search valid alternatives to conventional anthelmintic drugs is growing. Plants appear to be a source of compounds called secondary metabolites or allelochemicals, including terpenoids, alkaloids, glycosides, flavonoids, coumarins, quinones, saponins, and phenolic compounds. They do not have an apparent role in plant life but really play an important function such as defend itself from herbivores or pathogens. Moreover, many secondary metabolites exhibit a biological activity such as negative effects on worm biology (Heisey, 1997; Hoste and Torres-Acosta, 2011). Consequently, a large number of plants or plant extracts have been

investigated for their anthelmintic activities. Kamaraj et al. (2011) studied the efficacy of leaf, bark and seed extracts of selected plants against *H. contortus* in sheep using the egg hatching test and the larval development assay. Many studies were conducted to evaluate anthelmintic activity of selected plants of *Meliaceae* family such as *Melia azedarach* L. and *Khaya senegalensis*. Drupe extracts of *M. azedarach* L. showed anthelmintic activity against *Bunostomum trigonocephalum* and *Oesophagostomum columbianum* (Szewczuk et al., 2006). Moreover, the seed ethanol extract of *M. azedarach* L. revealed an interesting activity against *H. contortus* eggs while leaf ethanol extract showed in the larval development test a lethal concentration 50 (LC_{50}) of 9.18 mg/mL (Maciel et al., 2006). *In vitro* and *in vivo* studies were conducted to evaluate anthelmintic effects of *K. senegalensis* against GIN in sheep. Ethanolic and aqueous extracts was tested *in vitro* and nematicidal activity was evaluated through larval development test. Whereas, *in vivo* studies was conducted administering orally in sheep the ethanolic extract and monitoring the effects through FECRT. The anthelmintic activity of the extract could be attributed to tannins contained in the plant (Ademola et al., 2004). High concentrations of tannins are anti-nutritional because reduce nutrient digestibility. On the other hand, moderate concentrations or in mixture have benefice effects because in the rumen condensed tannins can bind to proteins through weak binding to form complexes that protect proteins against rumen degradation with the result of increasing of protein nutrition for the animal. This effect increase animal performance, improving sustainable productivity and reduce parasite infestations (Min and Hart, 2003; Frutos et al., 2004). Many studies analyzed tanniferous plants as possible alternatives to control GI infestations. Molan et al., (2003) studied the *in vitro* anthelmintic effects of monomer units of condensed tannins called 3-flavan-3-ols and their galloyl derivatives against *T. columbriformis*. Aerial part extracts of *Thymus capitatus* showed *in vitro* anthelmintic effects against *H. contortus;* the ethanolic extract showed higher activity against adult worms than the aqueous extract (Elandalousi et al., 2013).

8. *In vivo* sheep and goats studies

In literature, there are many *in vivo* studies focusing on the anthelmintic activity of plant extracts, essential oils or plant integrated in the diet. Small ruminants can be artificially or naturally infected by GIN, in most cases the studies were performed in indoors trials. Al-Shaibani et al., (2009) evaluated the anthelmintic activity of *Fumaria parviflora* against GIN through *in vitro* and *in vivo* studies. Ethanolic extract showed more *in vitro* ovicidal and larvicidal activity against *H. contortus* than the aqueous extract. The highest reduction on FWECs, monitored through *in vivo* grazing conditions, were obtained 14 days after a single oral dose of 200 mg/kg either of ethanolic and aqueous. On the other hand, anthelmintic efficacy of *Lippia sidoides* were investigated in sheep administrating growing doses of essential oil during 5 days and using a single dose of ivermectin as a positive control. The study was carried out in indoor conditions and fecal egg counts reduction test was used to evaluate the efficacy (Camurça-Vasconcelos et al., 2008). Anthelmintic effects of condensed tannins were studied administrating quebracho extracts to goats infected experimentally with *H. contortus* and reared indoors. Quebracho was obtained by barks of a tropical tree *Schinopsis* spp. At the end of the study a reduction of egg excretion were observed through a decreasing of female fecundity (Paolini et al., 2003).

9. References

Ademola I. O., Fagbemia B. O., Idowub S. O. 2004. Evaluation of the anthelmintic activity of *Khaya senegalensis* extract against gastrointestinal nematodes of sheep: *in vitro* and *in vivo* studies. *Vet. Parasit.*, 122, 151–164.

Al-Shaibani I. R. M., Phulan M. S., Hiekh M. S. 2009. Anthelmintic activity of *Fumaria parviflora* (Fumariaceae) against Gastrointestinal Nematodes of Sheep. *Int. J. Agric. Biol.*, 11, 431–436.

Camurça-Vasconcelos A. L. F., Bevilaqua C. M. L., Morais S. M., Maciel M. V., Costa C. T. C., Macedo I. T. F., Oliveira L. M. B., Braga R. R., Silva R. A., Vieira L. S., Navarro A. M. C. 2008. Anthelmintic activity of *Lippia sidoides* essential oil on sheep gastrointestinal nematodes. *Vet. Parasit.*, 154, 167–170.

Chartier C., Pors I., Hubert J., Rocheteau D., Benoit C., Bernard N. 1998. Prevalence of anthelmintic resistant nematodes in sheep and goats in Western France. *Small Ruminant Res.,* 29, 33-41.

Coles G. C., Jackson F., Pomroy W. E., Prichard R.K, Von Samson-Himmelstjerna G., Silvestre S., Taylor M. A., Vercruysse J. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet. Parasit.*, 136, 167–185.

D.M. 4 marzo 2005. Elenco dei medicinali per uso veterinario.

Domke A. V. M., Chartier C., Gjerde B., Höglund J., Leine N., Vatn S., Stuen S. 2012. Prevalence of anthelmintic resistance in gastrointestinal nematodes of sheep and goats in Norway. *Parasitol. Res.,* 111, 185–193.

Elandalousi R. B., Akkari H., B'chir F., Gharbi M., Mhadhbi M., Awadi S., Darghouth M. A. 2013. *Thymus capitatus* from Tunisian arid zone: chemical composition and *in vitro* anthelmintic effects on *Haemonchus contortus*. *Vet. Parasitol.*, 197, 374-378.

European Medicines Agency, 2012. European public MRL assessment report (EPMAR). Monepantel (caprine and ovine species). EMA/CVMP/165324/2008.

Europe Medicines Agency, 2012. Withdrawal extension assessment report. EMA/794474/2012.

Frutos P., Hervás G., Giráldez F. J. and Mantecón A. R. 2004. Review. Tannins and ruminant nutrition. *Spanish Journal of Agricultural Research*, 2, 191-202.

Gasser R. B. 2006. Molecular tools-advances, opportunities and prospects. *Vet. Parasitol.*, 136, 69–89.

Heisey R. M., 1997. Allelopathy and the secret life of *Ailanthus altissima*. *Allelopathy*, 57, 28-36

Hoste H., Chartier C. 1998. Response to challenge infection with *Haemonchus contortus* and *Trichostrongylus colubriformis* in dairy goats. Consequences on milk production. *Vet. Parasitol.*, 74, 43–54.

Hoste H., Torres-Acosta J. F. J. 2011. Non chemical control of helminths in ruminants: Adapting solutions for changing worms in a changing world. *Vet. Parasitol.*, 180, 144– 154.

Kamaraj C., Rahuman A. A, Elango G., Bagavan A., Zahir A. A. 2011. Anthelmintic activity of botanical extracts against sheep gastrointestinal nematodes, *Haemonchus contortus*. *Parasitol. Res.,* 109*,* 37-45.

Kaminsky R., Gauvry N., Schorderet Weber S., Skripsky T., Bouvier J., Wenger A., Schroeder F., Desaules Y., Hotz R., Goebel T., Hosking B. C., Pautrat F., Wieland-Berghausen S., Ducray P. 2008. Identification of the amino-acetonitrile derivative monepantel (AAD 1566) as a new anthelmintic drug development candidate. *Parasitol. Res.*, 103, 931-939.

Karadzovska D., Seewald W., Browning A., Smal M., Bouvier J., Giraudeul J.M. 2009. Pharmacokinetics of monepantel and its sulfone metabolite, monepantel sulfone, after intravenous and oral administration in sheep. *J. Vet. Pharmacol Ther*. 32, 359–367.

Maciel M. V., Morais S. M., Bevilaqua C. M., Camurça-Vasconcelos A. L., Costa C. T., Castro C. M. 2006. Ovicidal and larvicidal activity of *Melia azedarach* extracts on *Haemonchus contortus*. *Vet. Parasitol.*, 140, 98-104.

Makovcová K., Langrová I., Vadlejch J., Jankovská I., Lytvynets A., Borkovcová M. 2008. Linear distribution of nematodes in the gastrointestinal tract of tracer lambs. *Parasitol Res.*, 104, 123-6.

Mandonnet N., Ducrocq V, Arquet R, Aumont G. 2003. Mortality of Creole kids during infection with gastrointestinal strongyles: a survival analysis. *J. Anim. Sci.,* 81, 2401-2408.

McKellar Q. A., Jackson F. 2004. Veterinary anthelmintics: old and new. *Trends in parasitology*, 20, 456-461.

Min B. R., Hart S. P. 2003. Tannins for suppression of internal parasites. *J. Anim. Sci.*, 81, 102-109.

Ministero della Salute, GU n.131 del 7-6-2012. Modificazione dell'autorizzazione all'immissione in commercio del medicinale per uso veterinario «Valbazen».

Molan A. L., Meagher L. P., Spencer P A., Sivakumaran S. 2003. Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. *Int. J. Parasitol.*, 14, 1691-1698.

Panuska C. 2006. Lungworms of ruminants. *Vet. Clin. N. Am. Food Al. Practice*, 22, 583-593.

Paolini V., Bergeaud J. P., Grisez C., Prevot F., Dorchies Ph., Hoste H. 2003. Effects of condensed tannins on goats experimentally infected with *Haemonchus contortus*. *Vet. Parasitol.*, 113, 253–261.

Riviere J. E., Papich M. G. 2009. Veterinary Pharmacology and Therapeutics. Ninth edition. Wiley Black Well, Iowa, USA.

Roeber F., Jex A. R., Gasser R. B. 2013. Advances in the diagnosis of key gastrointestinal nematode infections of livestock, with an emphasis on small ruminants. *Biotechnol .Adv.*, 31, 1135-52.

Sargison N. D. 2008. Sheep Flock Health: A Planned Approach. 1st ed. Blackwell, Oxford, UK.

Sargison N. D. 2011. Pharmaceutical control of endoparasitic helminth infections in sheep. *Vet.Clin. North Am. Food Anim. Pract.*, 27,139-156.

Sargison N. D. 2012. Pharmaceutical treatments of gastrointestinal nematode infections of sheep-future of anthelmintic drugs. *Vet. Parasitol.,*189, 79-84.

Scala A. 2006. Meccanismi fisiopatogenetici dei principali tricostrongilidi abomasali dei piccoli ruminanti. *Parassitologia*, 48, 403-408.

Sechi S., Giobbe M., Sanna G., Casua S., Carta A., Scala A. 2010. Effects of anthelmintic treatment on milk production in Sarda dairy ewes naturally infected by gastrointestinal nematodes. *Small Ruminant Res.*, 88, 145–150.

Southwell J., Fisk C., Sallur N. 2008. Internal Parasite Control in Sheep. Reference Manual. 1st ed. Deborah Maxwell, DPI&F, Queensland.

Sutherland I., Scott I. 2010. Gastrointestinal Nematodes of Sheep and Cattle: Biology and Control. 1st ed. Blackwell, Chichester, UK.

Szewczuk, V. D., Mongelli, E. R., Pomilio, A. B. 2006. *In vitro* anthelmintic activity of *Melia azedarach* naturalized in Argentina. *Phytother. Res.*, 20, 993-996.

Taylor M. A., Coop R. L., Wall R. L. 2007. Veterinary Parasitology. 3rd ed. Blackwell Publishing, Oxford, UK.

Taylor, M. A., Hunt, K. R., Goodyear, K. L. 2002. Anthelmintic resistance detection methods. *Vet. Parasit. ,*3, 183-194.

Thienpont D., Vanparijs O. F., Raeymaekers A. H., Vandenberk J., Demoen J. A., Allewijn F. T., Marsboom R. P., Niemegeers C. J., Schellekens K. H., Janssen P. A. 1966. Tetramisole (R 8299), a new, potent broad spectrum anthelmintic. *Nature*, 12, 1084-1086.

Torres-Acosta J. F. J., Hoste H. 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. *Small Ruminant Res.*, 77, 159–173.

Torres-Acosta J. F. J., Mendoza-de-Gives P., Aguilar-Caballero A. J., Cuellar-Ordaz J. A. 2012a. Anthelmintic resistance in sheep farms: Update of the situation in the American continent. *Vet. Parasit.*, 189, 89– 96.

Torres-Acosta J. F. J., Sandoval-Castro C. A., Hoste H., Aguilar-Caballero A. J., Cámara-Sarmiento R., Alonso-Díazc M. A. 2012b. Nutritional manipulation of sheep and goats for the control of gastrointestinal nematodes under hot humid and subhumid tropical conditions. *Small Ruminant Res.*, 103, 28– 40.

Veneziano, V., Rubino, R., Fedele, V., Rinaldi, L., Santaniello, M., Schioppi, M., Cascone, C., Pizzillo, M., Cringoli, G. 2004. The effects of five anthelmintic treatment regimens on milk production in goats naturally infected by gastrointestinal nematodes. *S. Afr. J. Anim. Sci.*, 34, 251-253.

Zanzani S. A., Gazzonis A. L., Di Cerbo A., Varady M., Manfredi M. T. 2014. Gastrointestinal nematodes of dairy goats, anthelmintic resistance and practices of parasite control in Northern Italy. *BMC Vet. Res.*, 10:114.

http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Ivermectin_35/WC500060250.pdf

http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014674.pdf

Aims of the dissertation

The resistance of the gastrointestinal nematodes to conventional anthelmintic drugs is a global issue that involve small ruminant herds management. In the last years, plant extracts and their secondary metabolites are considered a valid alternative to control the nematode infestations. Taking into account these information, aim of the chapter 2 was the evaluation of the *in vitro* activity of *R. chalepensis* L., and *Ailanthus altissima* extracts and of selected phytochemicals (2-undecanone, (E,E)-2-4-decadienal, furfural, 2-hydroxybenzaldehyde and epigallocatechin gallate) against gastrointestinal nematodes and lungworms in small ruminants. Considering the EC_{50} values obtained *in* the *vitro* assay, in the chapter 3 we reported experiments with increasing doses of furfural and 2-hydroxybenzaldehyde orally administered to Sarda dairy ewes. The health status of the animals and the effects on their diet utilization were monitored. Moreover, the potential carry over into milk of these compounds and the residues in urine and faeces were investigated. In addition, the potential toxicological effects on the ewes causing by furfural and 2-hydroxybenzaldehyde were studied. On the other hand, in the chapter 4 we reported the *in vivo* anthelmintic activity of furfural, 2-hydroxybenzaldehyde and 2-undecanone against gastrointestinal nematodes. The carry over into milk of compounds administered or their metabolites were studied and the potential toxicological effects on ewes were monitored.

CHAPTER 2

In vitro anthelmintic activity of selected aldehydes and ketones of botanical origin against gastrointestinal nematodes and lungworms of small ruminants

Elisa Ortu

In *vitro* anthelmintic activity of selected aldehydes and ketones of botanical origin against gastrointestinal nematodes and lungworms of small ruminants

Abstract. Nematode infestations cause annually important economic losses to livestock production in small ruminants such as reduction of milk and meat yield. Because the resistance of parasites to conventional anthelmintic products is growing, studies evaluating the effectiveness of alternative products against gastrointestinal nematodes and lungworms are needed. This study was carried out to evaluate the *in vitro* nematicidal activity of two plant extracts, *Ruta chalepensis* L. and *Ailanthus altissima*, and some aldehydes of plant origin such as furfural, 2-hydroxybenzaldehyde, (E,E)-2-4-decadienal and ketones such as 2-undecanone against gastrointestinal nematodes. Moreover, was assessed the *in vitro* efficacy of furfural and 2-hydroxybenzaldehyde against freshly first-stage larvae (L_1) of lungworms and the activity of epigallocatechin gallate against gastrointestinal nematodes. For what concern gastrointestinal nematodes, faecal samples were collected from the rectum of Sarda dairy ewes and worm eggs were identified by Mc Master technique. The third stage larvae (L_3) of Strongyle type nematodes were obtained by coproculture and larvae were identified using an optic microscope 40x. On the other hand for the lungworms study, faeces were collected from the rectum of dairy goats and L_1 were recovered according to the Baermann technique. The larvae were identified with an optic microscope 40x especially for *Muellerius capillaris* (90%) and *Neostrongylus linearis* (10%). The larval assay, by using Cellstar 96-well cell culture plates, was performed to evaluate the effects of the plant extracts and the phytochemicals against L_3 and L_1. 2-undecanone and (E,E)-2-4-decadienal showed the highest nematicidal activity against gastrointestinal nematodes with a $EC_{50/24h}$= 0.88 and 1.03 mg/mL respectively, while for furfural, 2-hydroxybenzaldehyde and epigallocatechin gallate this concentration reference was 1.83 and 2.19 mg/mL at 24h and 1.79 mg/mL at 48h, respectively. *R. chalepensis* L. methanol extract showed the highest anthelmintic activity with an EC_{50}= 0.10 ± 0.06 mg/mL after 96h from treatment, while the *R. chalepensis* L. essential oil an EC_{50}= 1.20 ± 0.03 mg/mL after 48h from treatment. On the contrast, *A. altissima* wood methanol extract did not show relevant anthelmintic activity against gastrointestinal nematodes. In L_1 lungworms, 2-

hydroxybenzaldehyde showed higher activity than furfural. The highest nematicidal activity was for 2-hydroxybenzaldehyde after 48 hours with EC_{50} = 0.09 ± 0.05 mg/mL, while furfural exhibited the highest activity after 24 hours with EC_{50}= 1.70 ± 1.27 mg/mL. The experimental data revealed *in vitro* dose-dependent anthelmintic activity. However, the *in vitro* effects against gastrointestinal nematodes have to be carefully evaluated under *in vivo* conditions.

1. Introduction

Helminthiasis is one of the major small ruminant health issue with a strong impact on breeding performance. This issue limits productivity causing important economic losses such as reduction of milk and meat production, retarded growth, impaired fertility and in cases of high infestations the death of the host (Niekerk and Pimentel, 2004). In modern farming systems the control of nematode infestations commonly is achieved by regular use of anthelmintics but the consequence of their wide and indiscriminate use is causing the development of resistance of gastrointestinal nematodes (GIN) in small ruminants. In the last years, many studies confirmed the potential nematicidal activity of many plants and plant extracts against gastrointestinal nematodes (Cala et al., 2012; Al-Shaibani et al., 2009; Iqbal et al., 2006). The use of secondary metabolites of botanical origin could be a good and a valid alternative to the treatment of helminthiasis. Many plants produce secondary metabolites or allelochemicals, that have no apparent role but play important functions such as the defence of the plant by herbivores (reducing palatability), pathogen invasions (antimicrobial effects) or inhibit the plant growth (Heisey, 1997). Kamaraj et al. (2011) studied the *in vitro* efficacy of leaf, bark and seed extracts of selected plants against *Haemonchus contortus* in sheep. Methanol extracts of *A. paniculata*, *A. squamosa*, *D. metel* and *S. torvum* showed 100% of egg hatching at 25 mg/mL, while the larval development assay indicated for *A. squamosa* and *S. torvum* an EC_{50} of 3.53 and 3.71 mg/mL, respectively. Moreover, the drupe extracts of *Melia azedarach L.* showed a grater anthelmintic activity against *Bunostomum trigonocephalum* respect to piperazine phosphate and hexylresorcinol used as a positive controls. On the other hand, the same extracts showed less activity against *Oesophagostomum columbianum* respect to hexylresorcinol (Szewczuk et al., 2006). Other studies showed that the seed ethanol extract has an interesting activity against eggs of *H. Contortus* while the leaf ethanol extract showed a LC_{50} of 9.18 mg/mL in the larval development test (Maciel et al., 2006). The methanol extract obtained from the fruits possess nematicidal activity against root knot nematodes and their main constituents are 5-hydroxymethylfurfural and furfural (Ntalli et al., 2010). *Thymus capitatus* aerial part extracts showed anthelmintic effects against *H.*

contortus with an LC_{50} of 0.37 mg/mL for the ethanolic extract and 6.34 mg/mL for the aqueous extract (Elandalousi et al., 2013). *In vitro* and *in vivo* studies were conducted to evaluate anthelmintic effects of *Khaya senegalensis* against GIN in sheep. Ethanolic and aqueous extracts showed no significant differences *in vitro* with an LC_{50} of 0.51 and 0.69 mg/mL, respectively. Whereas, *in* the *vivo* assay, the ethanolic extract administrated by oral drench reduced the faecal worm egg counts (FWECs) of 88.8% (Ademola et al., 2004). Molan et al., (2003) studied the *in vitro* anthelmintic effects of monomer units of condensed tannins called 3-flavan-3-ols and their galloyl derivatives against *Trichostrongylus columbriformis*. Epigallocatechin gallate were more active than the other 3-flavan-3-ols in the egg hatch assay. The results obtained in this study indicated that the anti-parasite activity of condensed tannins depend on the number of hydroxyl groups present in the benzene ring. Ntalli et al. (2011) reported the anthelmintic activity of the essential oil and the methanol extract of *Ruta chalepensis* L. against root knot nematodes. *Ruta chalepensis* L. essential oil (REO) showed more activity then *Ruta chalepensis* L. methanol extract (RME), and 2-undecanone was considered the main component responsible for the nematicidal effect of the plant. *R.chalepensis* L. is a perennial herb growing up to 30-70 centimetres tall, belonging to Rutaceae family. In Italy, the plant is commonly known as rue or Ruta d'Aleppo. It is native to Mediterranean region but grows in many parts of the world, including Mexico, California, Africa and Saudi Arabia. The leaves are long and lanceolate containing oil glands and strong smelling. The flowers are cymes with 4-5 sepals and petals, 8-10 stamens and a superior ovary. Bright yellow petals form the inflorescence with rolled, fringed edges. The fruit is a glabrous capsule, which is divided into pointed lobes, containing brownish seeds. In the Mediterranean area the plant grow in walls, brush and mountain area. *R. chalepensis* L. is mentioned by Dioscorides for the treatment of nervous diseases (Stuart, 1979), while Cato the Censor in "*De Re Rustica*" reports that Rue (*R. Chalepensis* L. *and R. graveolens* L.) was used to nurse ulcers and sores. Moreover, these plants were used for their spiritual cleansings and magic properties (Menale and Muoio, 2007). In the Indian medicine is used for the treatment of dropsy, neuralgia, rheumatism and menstrual aches while in China is used as antivenom (Mansour Al Said et al., 1990). Vàsquez-Cruz et al. (2010) reported that the aqueous extract of leaves of *R. chalepensis* L. have antihypertensive effects in rats in

accordance with the use in folk medicine. Furthermore, *R. chalepensis* L. is known for its anti-inflammatory, analgesic, antipyretic, abortive and spasmolytic properties (Günaydin and Savci, 2005). Phytochemical screening of the aerial parts of the *R. chalepensis* L. revealed the presence of alkaloids, flavonoids, furanocoumarins, tannins, phenols, saponins, volatile oil, amino acids, sterols and triterpenes (Günaydin and Savci, 2005; Mansour Al-Said et al., 1990). Tounsi et al. (2011) studied the essential oil and fatty acid composition of different organs of the plant (leaves, flowers, stems and fruits), and indicated the presence in the oils of volatile compounds such as 2-undecanone, 2-nonanol and 2-dodecanone. Haddouchi et al. (2013) reported that the aerial parts of REO contained ketones, acyclic alkenes, monoterpene hydrocarbons, esters, sesquiterpenes and aldehydes, while 2-nonanone and 2-undecanone were the major constituents. Moreover, the aqueous extract of *R. chalepensis* L. showed activity in reducing the numbers of sweet potato whitefly *Bemisia tabaci* immature while the same extract was not detrimental for *B. tabaci* parasitoid, *Eretmocerus mundus* (Al-mazra'awin and Ateyyat, 2009). Essential oil of fruits and leaves showed inhibition effects against *H. contortus* egg hatching (Hussien et al., 2011). On the other hand, Caboni et al. (2012) studied the nematicidal activity of *Ailanthus Altissima* against the root-knot nematode *Meloidogyne javanica*, proving a good activity of wood extract and particularly the main constituent responsible of the activity presumptively was (E,E)-2-4-decadienal. *Ailanthus Altissima*, commonly known as tree of heaven, is a deciduous tree of the Simaroubaceae family. It is native to northeast, central China and Taiwan and was brought in Europe and United States during the 18th century. It was used extensively as a street tree during of the 19th century, becoming an invasive species, capable of colonizing disturbed areas. Knowing for its legendary tolerance of urban life, *A. Altissima* grows rapidly and is capable to reach heights between 17 and 27 meters. The versatility of its reproduction methods and the presence of phytotoxic compounds in roots and leaves may also explain the success of this plant. In addition, toxin exudation from roots may contribute to the aggressiveness and persistence of this plant in inhospitable habitats (Heisey, 1990 and 1997). Nowadays, roots, leaves and barks are used in traditional Chinese medicine for their astringent properties and as insect repellent (Heisey, 1997; Kee, 2000). Members of the Simaroubaceae family, including *Ailanthus* produce some bitter secondary metabolites, known as quassinoids; they derive

biosynthetically from the degradation of triterpenes. They exhibit herbicidal effects and activity against insects, fungi, virus and cancer cells (Morris, 1986; Heisey, 1997). Moreover, *Ailanthus* species are known for their activity against endoparasites (De Martino et De Feo, 2008). Barks contain high amounts of ailanthone, the major phytotoxic compound of quassinoid family that possess herbicide activity (Pedersini et al., 2011). Different studies reported the presence in the plant of various chemical compounds; the main constituents are quassinoids and alkaloids. *A. Altissima* wood extract (AWE) contains different substances such as (E,E)-2-4-decadienal, 2-undecenal, hexanal, furfural and nonanal (De Martino et De Feo, 2008; Caboni et al., 2012). In the leaves were isolated volatile compounds included alcohols, aldheydes, acids, esters, monoterpenes and sesquiterpenes (Masteli J. and Jerkovi I., 2002). Taking into account the nematicidal potential of these plants, aim of this work was to evaluate the anthelmintic activity of the essential oil and the methanol extract of *R. chalepensis* L. aerial parts against GIN. Moreover, and through their chemical characterization the main pure compounds constituents the plant were tested. In addition, the nematicidal potential of the *Ailanthus altissima* methanol extract and its single main constituents against GIN were investigated. Furthermore, were tested other compounds of botanical origin such as 2-hydroxybenzaldehyde and furfural against lungworms and GIN and epigallocatechin gallate against GIN.

2. Materials and methods

Chemicals

Standards of furfural (F), 2-hydroxybenzaldehyde (2-HBA), (E,E)-2-4-decadienal (2,4-DEC), 2-undecanone (2-UND), epigallocatechin gallate (ECG) and levamisole hydrochloride (HCl) of purity greater than 98%, as well as tween 20, sodium phosphate dibasic, sodium phosphate monobasic and methanol were purchased from Sigma-Aldrich (Milano, Italy). Methanol, ethanol and water used were high-performance liquid chromatography (HPLC) grade.

Extraction and Chemical Characterization

Plant Materials.

The aerial parts of *R. chalepensis* L. and wood of *A. Altissima* were collected in February 2014 at Cagliari, Italy and were dried in absence of light at room temperature. Later, they were kept in the dark in a paper bag until use. The botanical identity of the plants was verified and they were deposited in the Department of Life and Environmental Sciences, University of Cagliari, Italy. Dried aerial plant parts and wood (100g) were ground and extracted with methanol (1:10 w/v) and after 20 hours the samples were homogenized with a sonicator apparatus for 15 minutes, filtered through a Whatman n. 40 filter and centrifuged for 15 min at 13000 rpm according to Ntalli et al., (2011). One part of the extracts were used for the chemical characterization by GC-MS and LC-QTOF/MS analysis while a second part was used to the bioassay tests. On the other hand, the aerial parts of *R. chalepensis* were subjected to hydro-distillation for 4h after the mixture started boiling. The distillation system consisted in a heating mantle, a condenser and in a Clevenger apparatus. The essential oil was extracted in hexane and was dried over anhydrous $MgSO_4$ to remove dissolved water. After filtration through a Whatman n. 40 filter the hexane was evaporated at low temperature under low pressure using a rotavapor Büchi (Sigma Aldrich, Milano, Italy). The essential oil obtained and the methanol extracts were kept in dark glass vials at -20°C until use. The yield of the essential oil and the extracts was determined on average over three replicates.

GC-MS Analysis.

The REO, RME and AWE were diluted (1:100 and 1:10 v/v, respectively) with hexane and injected for GC-MS analysis using a method according to Ntalli et al., (2011) and Caboni et al., (2012) with slight modifications. The chromatographic separation and identification of the main components were performed on a Trace GC ultra gas chromatograph (Thermo Finnigan, San Jose, CA) coupled with a Trace DSQ mass spectrometry detector, a split-splitless injector an Xcalibur MS platform. The column used was polar fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30 m x 0.25 mm; film thickness, J&W 0.25 μm Scientific Fisons, Folsom (CA). The injector and transfer line were at 250 °C, while the oven operating conditions were as follows: from 50°C (held for 5 min) to 280°C at 9°C/min heating rate and kept at this temperature 8 for min. The carrier gas was helium with a constant flow rate of 1 mL/min; the sample (0.20 μL) was injected in splitless mode (60 s). Mass spectrometry acquisition was carried out using the following conditions: ionization voltage, 70 eV; scan rate 1.6 scan/s; mass range, 50-550 amu. REO, RME and AWE components were identified by comparison of their retention times and mass fragmentation with those of authentic standards and computer matching against a NIST98 commercial library (99%), as well as retention indices as calculated according to Kovats, for alkanes C9-C24 compared with those reported by Adams (Adams, 2007). Moreover, concentration of essential oil components were expressed as area percent while in RME and AWE were only identified the main constituents.

LC-MS-Q-TOF Analysis.

RME and AWE were analysed using an Agilent HPLC 1200 series instrument equipped with a 6520 QTOF detector. The chromatographic separation was performed on a Varian column Pursuit XRS3 C8 (50 x 2.0 mm, 5 μm). The mobile phase consisted on (A) aqueous formic acid 0.1%, and (B) acetonitrile. The solvent gradient (*v/v*) was started from 10 % B and reaching 100 % B in 10 min, maintaining 100% B for 10 min for a total run of 20 min. RME and AWE were diluted 1:10 *v/v* with methanol and then 4μL of these solutions were injected into the electrospray interface (ESI) operating in the positive-ion detection mode. Mass spectral data were acquired in the range m/z 100-

1500, with an acquisition rate of 1.35 spectra/s, averaging 10000 transients. The source parameters were as follows: drying gas temperature 250 °C, drying gas flow rate 5 L/min, nebulizer pressure 45 psi, and fragmentor voltage 200 V. Data acquisition and processing were done using Agilent Mass Hunter Workstation Acquisition v B.02.00 software.

Effect of R. chalepensis L., A. Altissima extracts and pure constituent compounds against L_3 of GIN.

Faecal samples uncontaminated by soil or bedding were collected from the rectum of Sarda dairy ewes and kept at 4 °C until use. The egg counts and the identification of gastrointestinal strongyles were performed according to a modified McMaster technique (Raynaud, 1970; Rossanigo and Gruner, 1991). The third stage larvae (L_3) of Strongyle type nematodes were obtained by coproculture (Sargison, 2008): 50 g of faeces were kept in a perforated plastic bag during ten day upon low humidity conditions. After this period of incubation, faeces were suspending in water and larvae were collected through filtration in a Baermann apparatus. Larvae treated with Lugol's iodine solution were identified with an optic microscope as the following GIN species: *Teladorsagia* spp. (51,5%), *Haemonchus contortus* (24,7 %) and *Trichostrongylus spp.* (23,8%). RME, AWE and REO were tested against L_3 GIN at the dose range of 0.02-0.20 mg/mL, 0.003-0.03 mg/mL and 0.40-6.30 mg/mL, respectively and EC_{50} were calculated. The pure compounds 2-UND, 2-4-DEC and F, contained in *R. chalepensis* L. and *A. Altissima* were tested individually at the concentration ranges of 0.10-3.90 mg/mL for EC_{50} calculation. The stock solutions of RME and AWE were prepared in distilled water containing the polysorbate surfactant 20 (tween 20) while the stock solution of REO was prepared in ethanol and successively diluted with water containing tween 20. Moreover, stock solutions of 2-UND, 2-4-DEC and F were prepared using distilled water containing tween 20 and to overcome insolubility was used methanol. Therefore, these solvents were used both as carrier in wells treated and as control for death/paralysis correction in no treated wells. Final concentrations of solvent and tween 20 in only treatment wells never exceeded 1 and 0.3% respectively. Stock solutions of levamisole-HCl were prepared using water and were used as positive control. The larval bioassays were performed in Cellstar 96-well cell culture plates (Greiner Bio-One) and

each treatment consisted of 25 L_3 per well. The paralysis test was replicated six times per experiment. L_3 were analyzed at 1, 24, 48 h for pure compounds and also 96 h after treatment for the extracts with the aid of an inverted microscope (Euromex, The Netherlands) at 40x. Nematodes were ranked into two categories: motile and immotile/paralyzed. The classification of the larvae into immotile/paralyzed was ascertained by absence of motility during an observation period of 6-8 seconds.

Anthelmintic activity of furfural and 2-hydroxybenzaldehyde against L_1 of lungworms.

Faeces were collected from the rectum of the goats and L_1 were recovered according to the Baermann technique. The larvae were identified with a microscope 40x especially for *M. capillaris* (90 %) and *N. linearis* (10 %). The anthelmintic activity was assessed through larval development test using Cellstar 96-well cell culture plates (Greiner Bio-One). Each treatment was represented by about 25 L_1 per well. Stock solutions of F and 2-HBA were prepared using a 0.1 M phosphate-buffered saline solution (PBS). Buffered solution was used as control for the correction of paralysis data while stock solutions of levamisole-HCl were used as positive control. Stock solutions of levamisole-HCl were prepared using water as solvent and were used in the range of 0.30-23.0 mg/mL. On the other hand, F and 2-HBA were tested against nematodes at dosages ranging from 0.40 to 6.70 mg/mL and from 0.02 to 0.15 mg/mL, respectively. The bioassays were performed adding 0.1 mL of stock solution to 0.1 mL of aqueous solution containing the L_1 in each well. Every treatment was replicated per experiment six times. Plates were covered with tin foil and kept in the dark at 25°C; L_1 were counted after 1, 24 and 48 hours of incubation using an inverted microscope (Euromex, The Netherlands) at 10x. Nematodes were ranked into two categories: motile and immotile/paralyzed. The classification of the larvae into immotile/paralyzed was ascertained by absence of motility during an observation period of 6-8 seconds.

Nematicidal activity of 2-hydroxybenzaldehyde and epigallocatechin gallate against L_3 of GIN.

Stock solutions of 2-HBA and ECG were prepared using aqueous 0.1 M PBS and to circumvent solubility issues methanol was used; the final concentration of solvent in each well never exceeded the 1% (*v/v*). Moreover, 2-HBA and ECG were tested against L_3 at dosages ranging from 0.35 to 6.00 mg/mL and from 0.70 to 5.45 mg/mL, respectively. Aqueous solution of PBS and methanol were used as negative control and stock solutions of levamisole-HCl as positive control. The larval bioassay were performed in Cellstar 96-well cell culture plates (Greiner Bio-One) and each treatment consisted of 25 L_3 per well. The paralysis test was replicated six times per experiment. L_3 were analyzed at 1, 24 and 48 h after treatment with the aid of an inverted microscope (Euromex, The Netherlands) at 40x and were ranked into two categories: motile and immotile/paralyzed. The classification of the larvae into immotile/paralyzed was ascertained by absence of motility during an observation period of 6-8 seconds.

Statistical analysis.

The percentages of L_3 and L_1 paralyzed during the bioassay tests were corrected by elimination of the natural death/paralysis in the negative control according to the Schneider Orelli formula: corrected %=[(mortality % in treatment - mortality % in control)/(100 - mortality % in control)]x 100 (Puntener, 1981). The corrected percentages of L_3 and L_1 treated were analyzed by Probit analysis for the determination of EC_{50} values using Minitab 16 software (Minitab Inc., State College, PA) and a probability of 0.05 was used. EC_{50} indicate the concentration of the compound required for 50% death/ paralysis after correction of percentages with natural death/ paralysis. The results were expressed as $EC_{50} \pm$ SD and as mean of paralysis % ± SD. Moreover, linear regression was used to evaluate dose-response relationship.

3. Results and Discussion

Chemical composition of R. chalepensis and A. altissima extracts.

Mass spectrometry coupled to gas chromatography was used for the chemical characterization of REO, RME and AWE which were injected directly without the need of derivatization or purification steps. According to GC-MS analysis, REO afforded α-pinene, sabinene, limonene, 2-nonanone, 2-nonanol, geijerene isomer, geijerene, 2-decanone, octyl acetate, 2-undecanone, 2-dodecanone and 2-tridecanone (Figure 1 and Table 1). Moreover, 2-nonanone and 2-UND were the predominant ones at 23.3% and 22.1% respectively, followed by limonene at 11.8 % and octyl acetate at 9.6%. According to other authors (Dob et al., 2008; Günaydin, and Savci, 2005; Ntalli et al.,2011; Ali et al., 2013; Haddouchi et al., 2013), 2-UND and 2-nonanone were the major constituent of REO. Also Mejri et al., (2010) and Tounsi et al., (2011) indicated 2-UND as predominant compound but followed by 2-decanone for the first and 2-nonanol for the second author. The oil yield was 1.09 ± 0.05% w/w expressed on a dry basis. Ntalli et al. (2011) indicated as yield of REO 0.36 ± 0.1 %, Tounsi et al. (2011) described yields in the range of 0.39-2.46% for leaves, flowers, stems and fruits, whereas Günaydin and Savci (2005) found a yield of 0.70%. The results obtained in this study are in accordance with the variability caused by different plant organs, the altitude, the climate, the soil when plants grow, the harvest period and the extraction technique used (Tounsi et al., 2011; Ntalli et al., 2011; Bouzidi et al., 2012). GC-MS analysis allowed to identified in REO twelve compounds consisting for 81.9 % of the total oil composition; other unknown substances were present at low concentrations (Table 1). Moreover, the chemical composition of RME by GC-MS was in accordance to REO analyzed by the same method. On the other hand, AWE analyzed by GC-MS showed a similar profile to extract studied by Caboni et al., (2012), where the main constituents were hexanal, (E,E)-2,4-decadienal, acid acetic and 5-hydroxymethylfurfural. Furthermore, mass spectrometry coupled to liquid chromatography was used to identify RME and AWE chemical profiles. RME chemical profile was in accordance to the extracts studied by Ntalli et al. (2011), where rutin, angelicin and 8-methoxypsoralen were the main constituents and by Günaydin and Savci (2005) where psoralen, chalepin and chalepensis were identified (Figure 2).

Contrary to AWE analyzed by GC-MS, in AWE analyzed by LC-MS-Q-TOF, were not detected aldehydes probably because of their high volatility and low ionization.

Effect of R. chalepensis, A. altissima extracts and pure constituent compounds against L_3 of GIN.

REO and RME were tested against L_3 of GIN at the dose range of 0.40-6.30 mg/mL and 0.02-0.20 mg/mL, respectively. Szewczuk et al. (2006) evaluated the death of the worms by their stimulation in hot water at 50°C. Instead, in this study, to evaluate the death or paralysis of the L_3 they were stimulated by exposure to microscope light in accordance to Gill et al. (1991). Moreover, the experiments were carried out in the dark (Gill et al., 1991) and at room temperature (Szewczuk et al., 2006). To overcome insolubility of some pure compounds methanol was used but the final concentration of solvent in each well never exceeded 1% (*v/v*) to avoid the risk that higher concentration was toxic to L_3. Larvae were considered immotile or paralyzed when there was not observable moving during 6-8 seconds in accordance with Martin and Le Jambre (1979) that observed larvae for five second consecutives. When REO and RME were tested a clear dose dependent effect was observed (Figure 3 and 4) and 24 hours after treatment an anthelmintic activity was evident with a paralysis % at the higher doses tested of 93.7% for REO and 81.2% for RME (Table 2 and 3). The EC_{50} values after 1 day of exposure in test solution for REO and RME were 1.07 ± 0.02 mg/mL and 0.10 ± 0.08 mg/mL, respectively. As a result, the RME showed highest nematicidal activity than REO; this result was confirmed 96 hours after treatment with an EC_{50} of 0.10 ± 0.06 mg/mL for RME and 1.35 ± 0.03 mg/mL for REO (Table 2). Moreover, at 96 hours RME inhibited the 94.6% of L_3 at the higher dosage, whereas for REO was 90.1% of paralysis (Table 3). Exist a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested, with a R^2 values at 96 hours of 0.981(figure 3) and 0.984 (figure 4) for REO and RME, respectively (Table 2). On the contrary, Ntalli et al. (2011) observed a major activity of REO respect to RME against second-stage juveniles of root knot nematodes. These different results can be explained by the different species involved and because of *R. chalepensis* is a source of other secondary metabolites not detected in this study, included furanocoumarins, alkaloids (Günaydin and Savci, 2005), flavonoids, tannins, saponins, sterols and triterpenes

(Mansour Al-Said et al., 1990). Probably, the major nematicidal activity of RME respect to REO could be caused by the synergic action of different compounds, including 2-undecanone and other compounds more abundant in RME respect to REO detected by LC-MS-Q-TOF which require further nematicidal studies. Furthermore, AWE were tested against L_3 at concentration ranging from 0.003 to 0.03 mg/mL no showing nematicidal activity. On the contrary, Caboni et al. (2012) observed an interesting nematicidal activity of AWE against root knot nematode *Meloidogyne javanica*. The absence of nematicidal activity against L_3 can be explained by different species studied respect to Caboni et al. and because low concentrations of extract tested were used. It was not possible tested higher doses of extract for not exceeded the 1% of total methanol present in wells. Stock solutions of levamisole-HCl were used as positive control with an EC_{50} values at 24 and 48 hours after treatment of 0.66 and 0.63 mg/L, respectively (Table 4). These results can be compared with Martin and Le Jambre, (1979) that obtained for levamisole-HCl an LC_{50} between 1.3 and 9.0 µg/mL against L_3 of *Ostertagia* spp. 24 hours after treatment. The EC_{50} obtained in this study was lower than the Martin data. This difference can be explained by the different species involved in the larvae testing. Kamaraj et al. (2011) and Maciel et al. (2006) used ivermectin as positive control in larval development tests against *H. contortus*. The EC_{50} of the plants studied by Kamaraj et al. were in the range of 3.51 and 4.26 mg/mL, whereas the EC_{50} for ivermectin was 2.8 µg/mL. On the other hand, leaf ethanol extract of *Melia azedarach* showed a LC_{50} value of 9.18 mg/mL while ivermectin at 0.32 µL/mL produced a inhibition of 79.7%. The results obtained indicate that plant extracts studied are less active than conventional anthelmintic drug used as positive control and suggest probably the need to enhance the nematicidal activity through modifications of the chemical structures. Among the different compounds identified in REO, RME and AWE by GC-MS were tested the pure aldehydes 2,4-DEC and F and the ketone 2-UND. Only these compounds were studied because revealed the mayor activity against root knot nematodes (Ntalli et al., 2011; Caboni et al., 2012). The three compounds tested showed nematicidal activity with a EC_{50} values at 24 hours after treatment of 1.83 ± 1.50, 0.88 ± 0.73 and 1.03 ± 0.79 mg/mL for F, 2-UND and 2,4-DEC, respectively (Table 4). 2-UND demonstrated the major anthelmintic activity that was confirmed at 48 hours with an EC_{50} value of 1.06 ± 1.06. Can be noted a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested,

with a R^2 values at 48 hours of 0.796 (Figure 5), 0.988 (Figure 6) and 0.924 (Figure 7) for F, 2-UND and 2,4-DEC, respectively. Moreover, at 48 hours Fl inhibited the 74.3% of L_3 at the higher dosage, 2-UND the 98.6%, whereas 2,4-DEC the 81.6% (Tables 5, 6 and 7).

Anthelmintic activity of furfural and 2-hydroxybenzaldehyde against L_1 of lungworms.

L_1 of lungworms are very susceptible to climate condition and to solvent presence. Many assays were carried out to obtain the optimal conditions for their survival. The main problem was obtain low % of paralysis in negative control, essential to correct the results obtained in the tests with the natural paralysis. Initially, stock solution of oxygen peroxide were used with the aim of increase the oxygenation of the larvae, but not interesting results were obtained. Moreover, methanol was used as solvent to reduce the problem of insolubility of the compounds tested but L_1 showed high sensibility to it. Later, PBS was used to overcome insolubility problems and to obtain a pH value optimal. Furthermore, Rose (1957) demonstrated that L_1 of *M. capillaris* are susceptible to bright sunlight, which killed the larvae in a short time and for this reason in this study, L_1 were kept in the dark. Finally, PBS, room temperature and dark conditions were used to obtain the best results of survival in the negative control and consequently it was possible apply nematicidal bioassays. The 2-HBA showed higher nematicidal activity than F with EC_{50} = 0.09 ± 0.05 mg/mL after 48 hours. While, F exhibited the highest activity after 24 hours after treatment with an EC_{50}=1.70 ± 1.26 mg/mL (Table 10). Moreover, can be found a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested, with a R^2 values at 48 hours of 0.928 (Figure 11) and 0.832 (Figure 12) for F and 2-HBA, respectively (Table 10). Stock solutions of levamisole-HCl were used as positive control with a 88.9% and 100% of paralysis at dosage of 14.64 mg/L after 24 and 48 hours after treatment (Table 11). On the other hand, at 48 hours F inhibited the 91.2% of L_1 at the higher dosage, while 2-HBA the 76.9% (Table 11).

Nematicidal activity of 2-hydroxybenzaldehyde and epigallocatechin gallate against L_3 of GIN.

2-HBA or salicylaldehyde is a characteristic component of buckwheat aroma (Janeš and Kreft, 2008; Janeš et al., 2009) and possess nematicidal activity against J2 of *M. incognita* (PhD thesis of Aissani 2012-2013). On the other hand, ECG is the ester of epigallocatechin and gallic acid, very abundant in white and green tea (*Camellia sinensis*). Moreover, it belongs to flavan-3-ol gallates that are constituents of condensed tannins units of a variety of plant species. This catechin possess *in vitro* ovicidal and larvicidal activity against *Trichostrongylus columbriformis* (Molan et al., 2003) 2-HBA exhibit highest activity 24 hours after treatment with an EC_{50}=2.19 ± 2.02 mg/mL, while ECG at 48 hours with an EC_{50}=1.79 ± 1.72 mg/mL (Table 4). Stock solutions of levamisole-HCl were used as positive control. Both compounds showed a linear relationship between the mean values of paralysis percentage and the log concentration of solution tested, with a R^2 values of 0.970 at 24 hours for 2-HBA (Figure 8), 0.887 (Figure 9) at 48 hours for ECG and 0.974 at 24 hours for 2-levamisole HCl (Figure 10), respectively. Moreover, at 24 hours 2-HBA inhibited the 93.5% of L_3 at the higher dosage, whereas ECG the 86.3% (Tables 8 and 9). The anthelmintic activity of ECG probably is causing by the ester bond through the molecule epigallocatechin and gallic acid because gallic acid was tested pure and no shown interesting nematicidal activity. Moreover, Molan et al. (2003) observed that ECG showed a higher nematicidal activity than epigallocatechin against L_3 of *T. columbriformis*.

4. Conclusions

The present investigation clearly indicated that overall RME, REO and pure compounds of botanical origin possess anthelmintic activity against GIN. Especially, 2-UND, 2,4-DEC and RME were the most active. Moreover, RME tested against GIN was more active than several other plant extracts previously studied by other authors (Kamaraj et al. 2011; Maciel et al., 2006; Elandalousi et al., 2013; Ademola et al., 2004). With regard to lungworms, the results obtained indicate that F and 2-HBA possessed a larvicidal activity, while 2-HBA was the most active. The use of phytochemials is an opportunity for the development of valid alternatives against nematode infestations in small ruminant herds. In conclusion, these compounds may find an application in the future as anthelmintics in veterinary practices. This preliminary study could be useful to perform further *in vivo* experiments. However, more studied are needed to evaluate the mode of action of these active compounds and to optimize the nematicidal activity through studies of their chemical structure.

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6. References

Adams, R. P. 2007. Identification of Essential Oil Components By Gas Chromatography/Mass Spectrometry. 4thed. *Allured: Carol Stream,* IL- USA.

Ademola I. O., Fagbemia B. O., Idowub S. O. 2004. Evaluation of the anthelmintic activity of *Khaya senegalensis* extract against gastrointestinal nematodes of sheep: in *vitro* and in *vivo* studies. *Vet. Parasitol.*, 122, 151–164.

Aissani N., PhD, 2012-2013. Nematicidal, antimicrobial and acaricidal activity of plant secondary metabolites. Department of Live and Environmental Sciences, Cagliari, Italy.

Ali A., Demirci B., Kiyan H. T., Bernier U. R., Tsikolia M., Wedge D. E., Khan I. A., Başer K. H. C., Tabanca, N. 2013. Biting deterrence, repellency, and larvicidal activity of *Ruta chalepensis* (Sapindales: Rutaceae) essential oil and its major individual constituents against mosquitoes. *J. Med. Entomol.*, 50, 1267-1274.

Al-mazra'awi M. S., Ateyyat M. 2009. Insecticidal and repellent activities of medicinal plant extracts against the sweet potato whitefly, *Bemisia tabaci* (Hom.:Aleyrodidae) and its parasitoid *Eretmocerus mundus* (Hym.: Aphelinidae). *J. Pest Sci.*, 82, 149-154.

Al-Shaibani I. R. M., Phulan M. S., Shiekh M. 2009. Anthelmintic activity of *Fumaria parviflora* (Fumariaceae) against Gastrointestinal Nematodes of Sheep. *Int. J. Agr. Biol.*, 11, 431-436.

Bouzidi M. A., Latrèche A., Attaoui I., Benabderrahmane M., Mehdadi Z., Benyahia M. 2012. Antibacterial Effect of the Essential Oils Extracted From *Ruta chalepensis* L. and *Ruta montana* L. *Journal of Life Sciences*, 6, 898-902.

Caboni P., G. Ntalli N., Aissani N., Cavoski I., Angioni A. 2012. Nematicidal activity of (E,E)-2,4-Decadienal and (E)-2-Decenal from *Ailanthus altissima* against *Meloidogyne javanica*. *J. Agr. Food Chem.*, 60, 1146−1151.

Cala A. C., Chagas A. C., Oliveira M. C., Matos A. P., Borges L. M., Sousa L. A., Souza F. A., Oliveira G. P. 2012. *In vitro* anthelmintic effect of *Melia azedarach* L. and *Trichilia claussenii* C. against sheep gastrointestinal nematodes. *Exp. Parasitol.*, 130, 98-102.

De Martino, L., De Feo V. 2008. Chemistry and biological activities of *Ailanthus altissima* Swingle a review. *Phcog. Rev.*, 4, 339-350.

Dob T., Dahmane D., Gauriat-Desrdy B., Daligault V. 2008. Volatile constituents of the essential oil of *Ruta chalepensis* L. subsp. angustifolia (Pers.) P. Cout. *J. Essent. Oil Res.*, 20, 306-309.

Elandalousi R. B., Akkari H., B'chir F., Gharbi M., Mhadhbi M., Awadi S., Darghouth M. A. 2013. *Thymus capitatus* from Tunisian arid zone: chemical composition and *in vitro* anthelmintic effects on *Haemonchus contortus*. *Vet. Parasitol.*, 197, 374-378.

Gill J. H., Redwin J. M., van Wyk J. A., Lacey E. 1991. Detection of resistance to ivermectin in *Haemonchus contortus*. *Int. J. Parasitol.*, 21,771-776.

Günaydin K., Savci S. 2005. Phytochemical studies on *Ruta chalepensis* (Lam.) Lamarck. *Nat. Prod. Res.*, 19, 203-210.

Haddouchi F., Chaouche T. M., Zaouali Y., Ksouri R., Attou A., Benmansour A. 2013. Chemical composition and antimicrobial activity of the essential oils from four Ruta species growing in Algeria. *Food Chem.*, 141, 253-258.

Heisey R. M., 1990. Evidence for allelopathy by tree of heaven *Ailanthus altissima*. *J. Chem. Ecol.*, 16, 2039-2056.

Heisey R. M., 1997. Allelopathy and the secret life of *Ailanthus altissima*. *Allelopathy*, 57, 28-36.

Hussien, J., Urgessa, K., Regassa, F., Jemal, A., Abajebel, S., Hussien, N.2011. Anthelmentic effects of the essential oil extracts of selected medicinal plants against *Haemonchus contortus*. *Int. J. Agric. Res.,* 6*,* 290-298.

Iqbal Z., Lateef M., Jabbar A., Ghayur M. N., Gilani A. H. 2006. *In vitro* and *in vivo* anthelmintic activity of *Nicotiana tabacum* L. leaves against gastrointestinal nematodes of sheep. *Phytother. Res*. 20, 46-48.

Janeš D., Kantarb D., Kreft, S., Prosen H. 2009. Identification of buckwheat *(Fagopyrum esculentum Moench)* aroma compounds with GC–MS. *Food Chem.,* 112, 120-124.

Janeš, D., Kreft, S. 2008. Salicylaldehyde is a characteristic aroma component of buckwheat groats. *Food Chem.*, 109, 293-298.

Kamaraj C., Rahuman A. A, Elango G., Bagavan A., Zahir A. A. 2011. Anthelmintic activity of botanical extracts against sheep gastrointestinal nematodes, *Haemonchus contortus*. *Parasitol. Res.,* 109*,* 37-45.

Kee C. H. 2000. The Pharmacology of Chinese Herbs. Second Edition. *CRC Press LLC*, Boca Raton, Florida.

Maciel M. V., Morais S. M., Bevilaqua C. M., Camurça-Vasconcelos A. L., Costa C. T., Castro C. M. 2006. Ovicidal and larvicidal activity of *Melia azedarach* extracts on *Haemonchus contortus*. *Vet. Parasitol.*, 140, 98-104.

Mansour S. Al-Said, Tariq M., Al-Yahya M. A., Rafatullah S., Ginnawi O. T., Ageel A. M. 1990. Studies on *Ruta chalepensis*, an ancient medicinal herb still used in traditional medicine. *Journal of Ethnopharmacology*, 28, 305-312.

Martin P. J., Le Jambre L. F. 1979. Larval paralysis as an in vitro assay of levamisole and morantel tartrate resistance in Ostertagia. *Veterinary Science Communications*, 3, 159-164.

Masteli J., Jerkovi I. 2002. Volatile Constituents from the Leaves of Young and Old *Ailanthus altissima* (Mill.) Swingle Tree. *Croat. Chem. acta*, 75, 189-197.

Menale B, Muoio R. 2007. Usi tradizionali di *Ruta chalepensis* L. (Rutaceae) in Campania. Delpinoa, 49, 45-51.

Mejri J., Abderrabba M., Mejri M. 2010. Chemical composition of the essential oil of *Ruta chalepensis* L: Influence of drying, hydro-distillation duration and plant parts. *Ind. Crop.and Prod.,* 32, 671-673.

Molan A. L., Meagher L. P., Spencer P. A., Sivakumaran S. 2003. Effect of flavan-3-ols on *in vitro* egg hatching, larval development and viability of infective larvae of *Trichostrongylus colubriformis*. *Int. J. Parasitol.*, 14, 1691-1698.

Morris P. 1986. Secondary Metabolism in Plant Cell Cultures. *Cambridge University Press*, Cambridge, UK.

Niekerk W. A., Pimentel P. L. 2004. Goat production in the smallholder section in the Boane district in Southern Mozambique. *S. Afr. J.Anim. Sci.*, 34, 123-125.

Ntalli N. G., Vargiu S., Menkissogluspiroudi U., Caboni P. 2010. Nematicidal Carboxylic Acids and Aldehydes from *Melia azedarach* Fruits. *J. Agr. Food Chem.*, 58, 11390–11394.

Ntalli N. G., Manconi F., Leonti M., Maxia A., Caboni P. 2011. Aliphatic Ketones from *Ruta chalepensis* (*Rutaceae*) Induce Paralysis on Root Knot Nematodes. *J. Agr. Food Chem.*, 59, 7098–7103.

Pedersini C., Bergamin M., Aroulmoji V., Baldini S., Picchio R., Pesce P.G., Ballarin L., Murano E. 2011. Herbicide activity of extracts from *Ailanthus altissima* (Simaroubaceae). *Nat. Prod. Commun.*, 5, 593-6.

Puntener W. 1981. Manual for field trials in plant protection. 2nd ed. Ciba G, Basel, Switzerland.

Raynaud J. P. 1970. Etude de l'efficacitè d'une technique de copro scopie quantitative pour le diagnostic de routine et le controle des infestations parasitaires des bovins, ovins, èquins et porcins. *Ann. Parasitol.*, 45, 321-342.

Rose J.H. 1957. Observations on the bionomics of the free-living first stage larvae of the sheep lungworm, *Muellerius capillaris*. *J. Helminthol.*, 31, 17-28.

Rossanigo C. E., Gruner L. (1991). Accuracy of two methods for counting eggs of sheep nematode parasites. *Vet. Parasitol.*, 39,115-121.

Sargison N. 2008. Sheep Flock Health: A Planned Approach. 1st ed. Blackwell, Oxford, UK.

Stuart M. (1979). The Encyclopedia of Herbs and Herbalism. 1st ed. Orbis Publishing Ltd., London. 256-257.

Szewczuk, V. D., Mongelli, E. R., Pomilio, A. B. 2006. *In vitro* anthelmintic activity of *Melia azedarach* naturalized in Argentina. *Phytother. Res.*, 20, 993-996.

Tounsi, M.S., Wannes, W.A., Ouerghemmi, I., Msaada, K., Smaoui, A., Marzouk, B. 2011. Variation in essential oil and fatty acid composition in different organs of cultivated and growing wild *Ruta chalepensis* L. *Ind. Crop. Prod.*, 33, 617-623.

Vázquez-Cruz B., Sampson-Lizardi N., Rosas-Benitez A., Segura-Cobos D., Amato D. 2010. Antihypertensive activity of *Ruta chalepensis* L. leaves. *Pharmacology online*, 3, 354-363.

http://webbook.nist.gov/

Table 1. Chemical composition of REO by GC-MS analysis listed in order to elution.

compound	Rt	RI	mol/wt	EI-MS characteristics m/z (amu) (abundance)	REO %
sabinene	9.70	976	136.24	93 (100%); 77 (38%); 91 (36%)	0.82
β-pinene	9.82	980	136.23	93 (100%); 69 (30%); 91 (30%)	0.74
unknown	10.08	-	-	-	0.84
limonene	11.02	1031	136.24	68 (100%); 67 (75%); 93 (70%)	11.77
2-nonanone	12.32	1091	142.24	58 (100%); 71 (85%)	23.25
2-nonanol	12.49	1098	144.25	55 (100%); 69 (70%)	1.18
unknown	12.57	-	-	-	0.94
unknown	13.12	-	-	-	0.60
geijerene isomer	13.19	1137	162.27	79 (100%); 94 (43%)	0.94
geijerene	13.34	1137	162.27	79 (100%); 94 (90%)	5.25
2-decanone	14.17	1192	156.27	58 (100%); 71 (45%) 45 (10%)	3.35
unknown	14.31	-	-	-	0.65
octylacetate	14.86	1211	172.27	56 (100%); 70 (90%) 84 (85%)	9.57
unknown	15.28	-	-	-	0.42
2-undecanone	15.91	1291	170.30	58 (100%); 71 (85%) 85 (80%)	22.05
unknown	16.02	-	-	-	0.96
unknown	16.07	-	-	-	1.94
2-dodecanone	17.00	1369	184.31	58 (100%); 71 (50%) 85 (15%)	2.00
2-tridecanone	17.45	1392	198.34	58 (100%); 71 (45%) 85 (10%)	0.96
unknown	17.93	-	-	-	2.42
unknown	18.92	-	-	-	0.78
unknown	19.76	-	-	-	0.41

Table 2. EC_{50} ± S.D. (n=6) and R^2 values of *Ruta chalepensis* L. against gastrointestinal nematodes calculated at 24h, 48h and 96h after treatment.

compound	24h		48h		96h	
	EC_{50} (mg/mL)	R^2	EC_{50} (mg/mL)	R^2	EC_{50} (mg/mL)	R^2
REO	1.07 ± 0.02	0.871	1.20 ± 0.03	0.916	1.35 ± 0.03	0.981
RME	0.10 ± 0.08	0.926	0.11 ± 0.09	0.930	0.10 ± 0.06	0.984

Table 3. Mean inhibition percentage ± S.D. (n=6) of REO and RME against gastrointestinal nematodes calculated at 24h, 48h and 96h after treatment.

(mg/mL)	24h	48h	96h
REO	**% paralysis**	**% paralysis**	**% paralysis**
6.32	93.72 ± 0.00	92.29 ± 0.00	90.95 ± 0.00
3.16	89.54 ± 5.50	86.76 ± 4.39	75.28 ± 6.43
1.58	81.28 ± 17.49	74.71 ± 11.80	47.80 ± 22.64
0.79	27.30 ± 2.48	32.97 ± 10.41	13.82 ± 11.47
0.40	13.72 ± 1.98	9.72 ± 2.55	0.15 ± 1.61
RME	**% paralysis**	**% paralysis**	**% paralysis**
0.20	81.19 ± 0.00	78.94 ± 7.07	94.61 ± 0.00
0.09	73.15 ± 9.37	49.75 ± 7.82	51.21 ± 13.13
0.05	22.96 ± 13.47	43.09 ± 3.30	24.80 ± 10.60
0.02	0.20 ± 0.05	0.30 ± 0.00	0.50 ± 0.00
Levamisole-HCl	**% paralysis**	**% paralysis**	**% paralysis**
0.02	97.13 ± 0.00	95.31 ± 0.00	93.62 ± 1.99
Tween 20	**% paralysis**	**% paralysis**	**% paralysis**
0.3%in methanol(<1%)	12.20 ± 2.97	8.10 ± 3.48	5.71± 2.74
0.3%in ethanol(<1%)	5.91 ± 1.29	7.16 ± 0.48	8.30 ± 1.12

Table 4. EC_{50} ± S.D. (n=6) and R^2 values of furfural (F), 2-hydroxybenzaldehyde (2-HBA), 2-undecanone (2-UND), (E,E) 2,4-decadienal (2,4-DEC), epigallocatechin gallate (ECG), levamisole-HCl (LEV-HCl) against gastrointestinal nematodes calculated at 1h, 24h and 48h after treatment.

	1h		24h		48h	
compound	**EC_{50} (mg/mL)**	**R^2**	**EC_{50} (mg/mL)**	**R^2**	**EC_{50} (mg/mL)**	**R^2**
F	2.28 ± 1.66	0.730	1.83 ± 1.50	0.901	2.18 ± 1.19	0.796
2-UND	4.04 ± 3.33	0.998	0.88 ± 0.73	0.762	1.06 ± 1.06	0.988
2,4-DEC	0.94 ± 1.24	0.981	1.03 ± 0.79	0.933	1.09 ± 0.88	0.924
2-HBA	3.10 ± 2.62	0.944	2.19 ± 2.02	0.970	2.89 ± 2.50	0.922
ECG	2.59 ± 1.37	0.828	2.10 ± 2.14	0.866	1.79 ± 1.72	0.887
LEV-HCl	0.004 ± 0.005	0.964	0.0007± 0.0007	0.974	0.0006 ± 0.001	0.847

Table 5. Mean inhibition percentage ± S.D. (n=6) of furfural against gastrointestinal nematodes calculated at 1h, 24h and 48h after treatment.

furfural	**1h**	**24h**	**48h**
(mg/mL)	**% paralysis**	**% paralysis**	**% paralysis**
3.07	64.00 ± 14.34	77.47 ± 8.46	74.34 ± 12.70
1.53	19.98 ± 18.57	45.13 ± 10.04	36.71 ± 11.13
0.39	6.52 ± 10.31	12.78 ± 11.48	32.66 ± 13.71
0.10	0.20 ± 5.59	13.34 ± 12.85	14.08 ± 14.42

Table 6. Mean inhibition percentage ± S.D. (n=6) of 2-undecanone against gastrointestinal nematodes calculated at 1h, 24 h and 48 h after treatment.

2-undecanone	**1h**	**24h**	**48h**
(mg/mL)	**% paralysis**	**% paralysis**	**% paralysis**
3.89	44.87 ± 12.77	88.62 ± 0.00	98.55 ± 1.99
1.94	34.91 ± 17.71	87.95 ± 1.63	78.67 ± 6.63
0.97	37.49 ± 19.55	73.70 ± 10.29	54.95 ± 21.55
0.49	9.68 ± 0.00	17.54 ± 12.85	22.89 ± 12.71

Table 7. Mean inhibition percentage ± S.D. (n=6) of (E,E)-2,4-decadienal against gastrointestinal nematodes calculated at 1h, 24 h and 48 h after treatment.

2,4-decadienal	1h	24h	48h
(mg/mL)	% paralysis	% paralysis	% paralysis
2.26	81.95 ± 7.49	85.66 ± 5.47	81.61 ± 5.29
1.13	65.55 ± 16.54	79.19 ± 10.41	76.94 ± 7.79
0.28	2.16 ± 4.97	4.83 ± 10.59	4.91 ± 15.88

Table 8. Mean inhibition percentage ± S.D. (n=6) of 2-hydroxybenzaldehyde against gastrointestinal nematodes calculated at 1h, 24h and 48h after treatment.

2-hydroxybenzaldehyde	1h	24h	48h
(mg/mL)	% paralysis	% paralysis	% paralysis
5.99	87.20 ± 0.00	93.56 ± 0.00	88.14 ± 0.00
2.99	64.20 ± 8.15	73.07 ± 13.68	51.59 ± 16.35
1.50	24.78 ± 12.44	41.89 ± 11.97	35.20 ± 17.97
0.37	5.43 ± 0.00	10.36 ± 12.85	11.07 ± 15.17

Table 9. Mean inhibition percentage ± S.D. (n=6) of epigallocatechin gallate against gastrointestinal nematodes calculated at 1h, 24 h and 48 h after treatment.

Epigallocatechingallate	1h	24h	48h
(mg/mL)	% paralysis	% paralysis	% paralysis
5.46	48.57 ± 15.89	86.63 ± 8.26	93.11 ± 0.00
2.77	49.10 ± 17.82	78.31 ± 5.76	83.25 ± 8.17
1.37	32.51 ± 16.79	56.72 ± 12.70	59.22 ± 5.98
0.68	0.00 ± 0.00	0.00 ± 0.00	3.37 ± 17.76

Table 10. EC_{50} ± S.D (n=6) and R^2 values of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) against lungworm L_1calculated at 1h, 24 h and 48 h after treatment.

compound	1h		24h		48h	
	EC_{50} (mg/mL)	R^2	EC_{50} (mg/mL)	R^2	EC_{50} (mg/mL)	R^2
F	2.09 ± 0.96	0.909	1.70 ± 1.26	0.876	2.81 ± 2.20	0.928
2-HBA	0.06 ± 0.06	0.895	0.09 ± 0.05	0.905	0.09 ± 0.05	0.832

Table 11. Mean inhibition percentage ± S.D. (n=6) of furfural and 2-hydroxybenzaldehyde against lungworm L_1 calculated at 1h, 24h and 48h after treatment.

(mg/mL)	1h	24h	48h
furfural	**% paralysis**	**% paralysis**	**% paralysis**
6.71	99.25 ± 1.46	100.00 ± 0.00	91.22 ± 6.79
3.36	95.61 ± 6.67	93.48 ± 5.47	71.23 ± 13.43
1.68	33.46 ± 18.69	38.08 ± 11.25	27.29 ± 17.58
0.84	6.65 ± 16.58	31.65 ± 10.80	21.77± 13.45
0.42	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2-hydroxybenzaldehyde	**% paralysis**	**% paralysis**	**% paralysis**
0.14	86.11 ± 12.94	85.94 ± 12.55	76.87 ± 17.67
0.07	75.63 ± 11.60	78.46 ± 18.49	76.22 ± 34.22
0.04	47.64 ± 17.38	12.87 ± 11.29	3.76 ± 17.95
0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Levamisole-HCl	**% paralysis**	**% paralysis**	**% paralysis**
0.015	100.00 ± 0.00	88.90 ± 1.86	100.00 ± 0.00

Figure 1. GC/MS chromatograms of *R. chalepensis* L. essential oil. Peaks: (1) α-pinene, (2) sabinene, (3) unknown, (4) limonene, (5) 2-nonanone, (6) 2-nonanolo, (7) unknown, (8) unknown, (9) geijerene isomer, (10) geijerene, (11) 2-decanone, (12) unknown, (13) octyl acetate, (14) unknown, (15) 2-undecanone, (16) unknown, (17) unknown, (18) 2-dodecanone, (19) 2-tridecanone, (20) unknown, (21) unknown, (22) unknown.

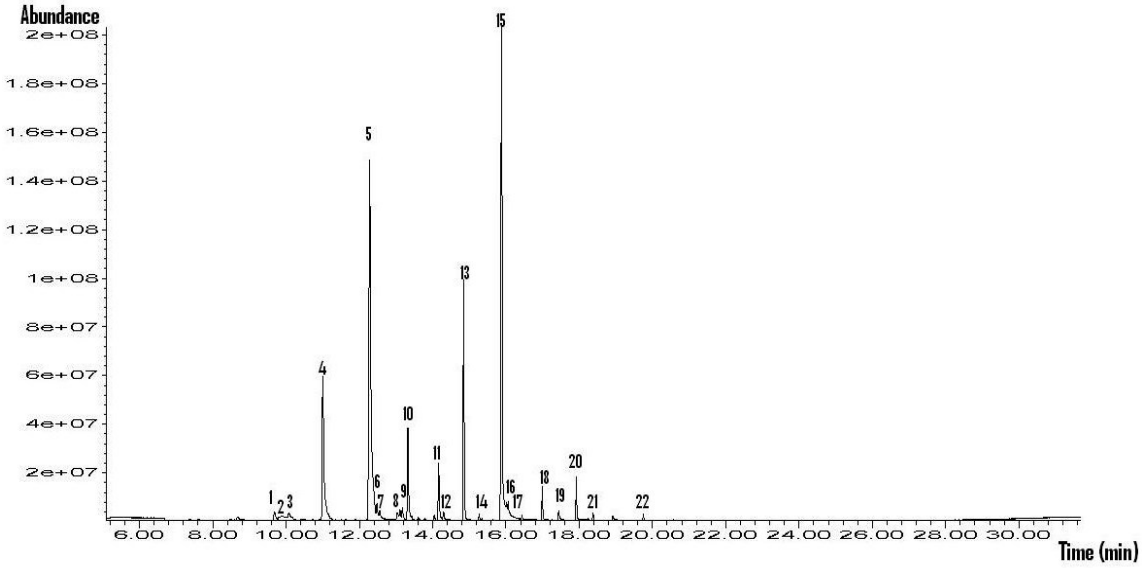


Figure 2. LC-MS-Q-TOF chromatograms of *R. chalepensis* L. methanol extract. Peaks: **(A)** psoralen, **(B)** chalepin, **(C)** chalepensis, **(D)** rutina, **(E)** angelicina and **(F)** methoxypsoralen.

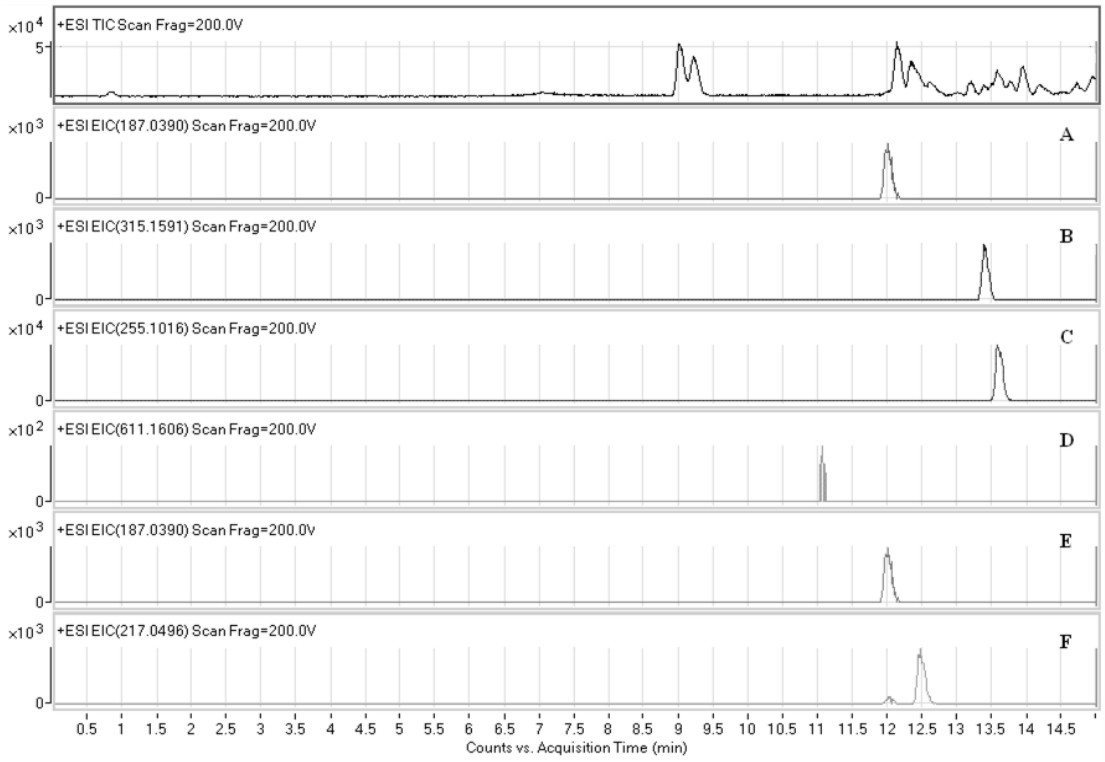


Figure 3. Relationship between the log concentration of REO and paralysis % of gastrointestinal nematodes at 24h (A), 48h (B) and 96h (C) after treatment.

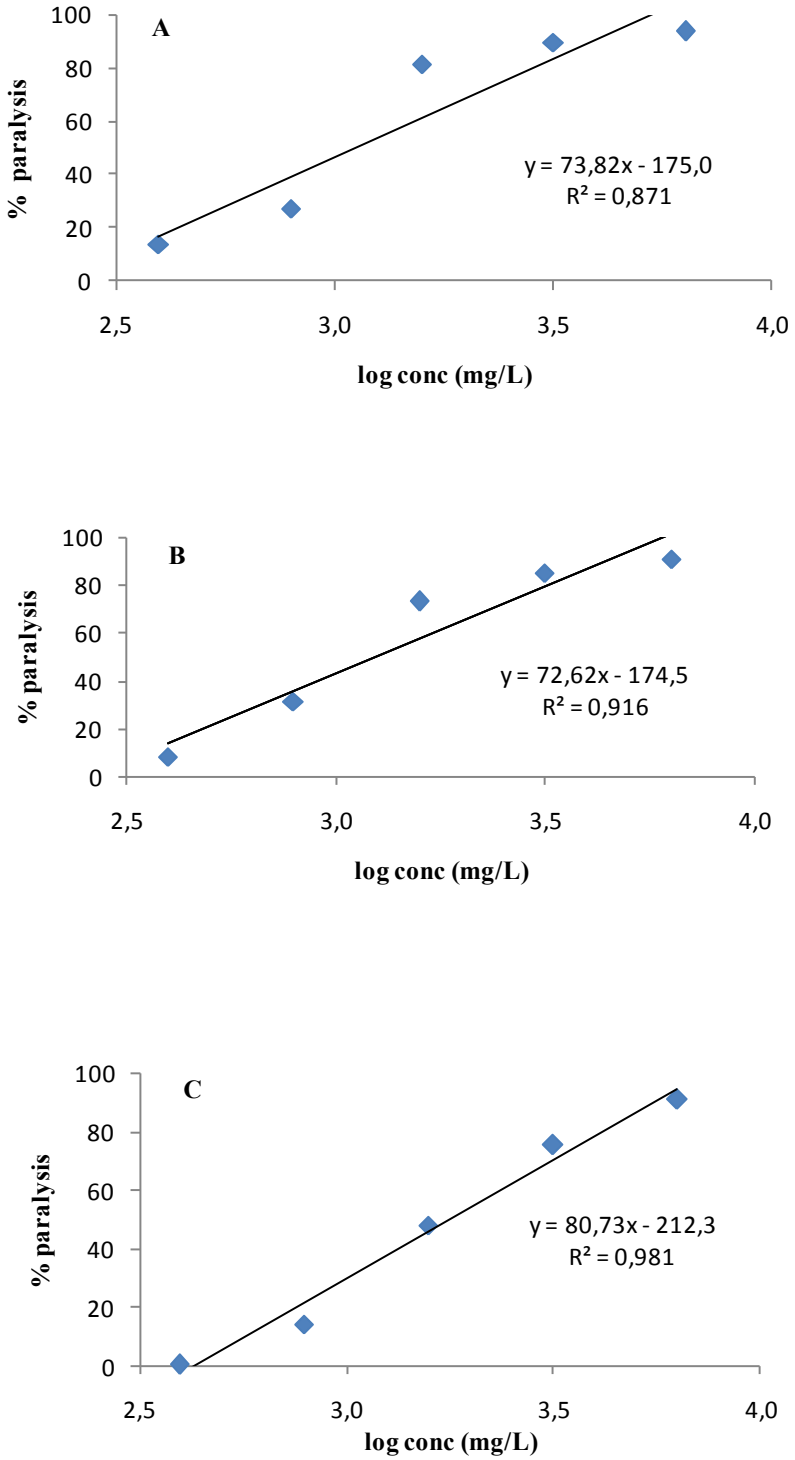


Figure 4. Relationship between the log concentration of RME and paralysis % of gastrointestinal nematodes at 24h (A), 48h (B) and 96h (C) after treatment .

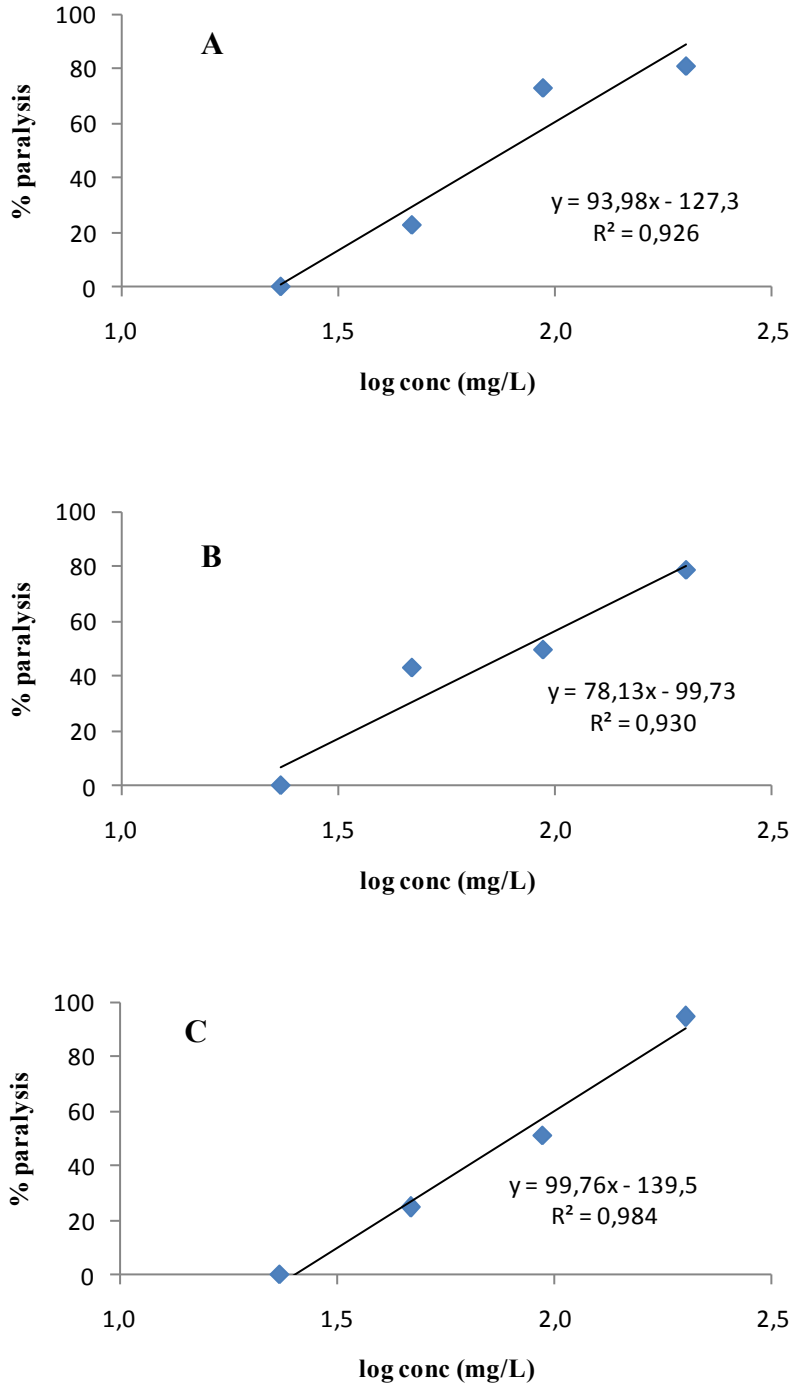


Figure 5. Relationship between the log concentration of furfural and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

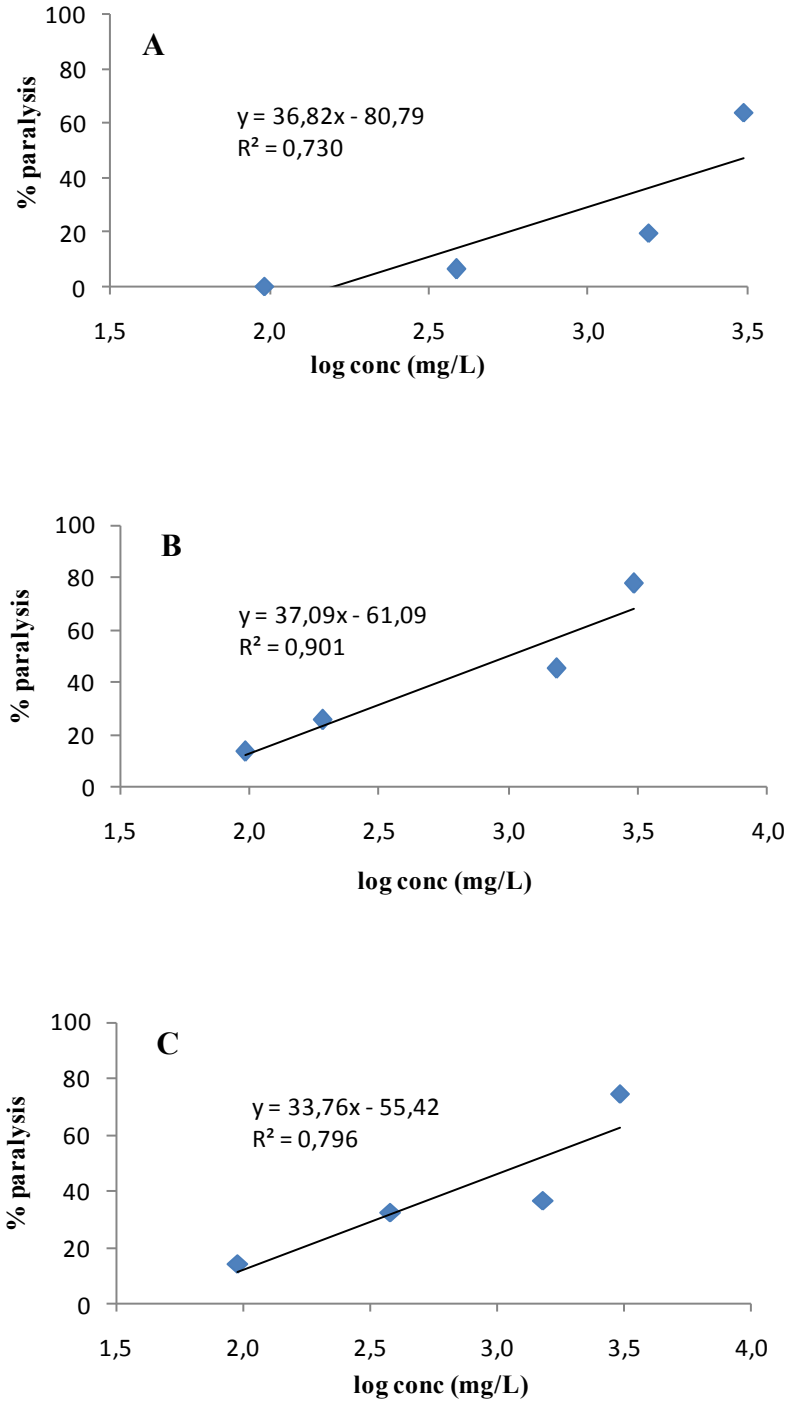


Figure 6. Relationship between the log concentration of 2-undecanone and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

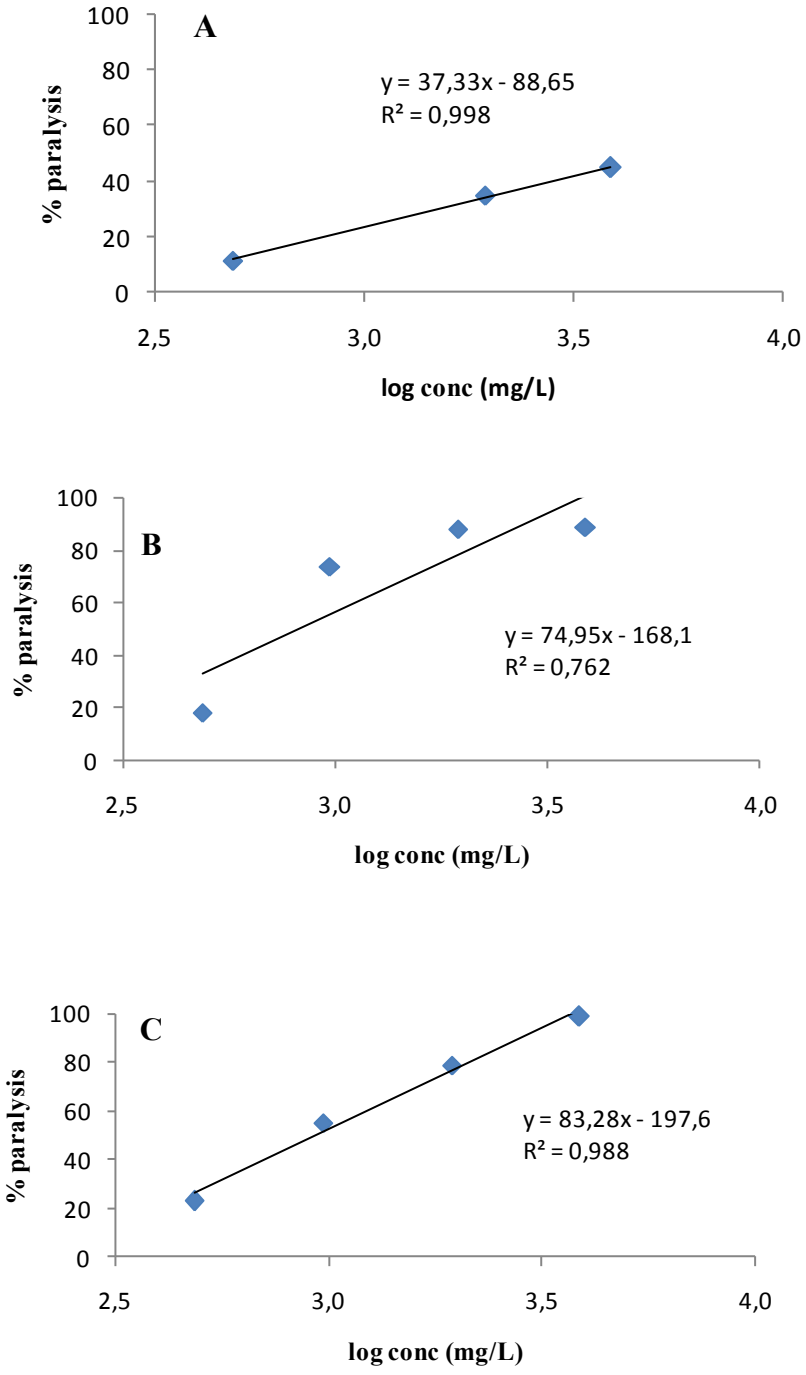


Figure 7. Relationship between the log concentration of (E,E) 2,4-decadienal and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

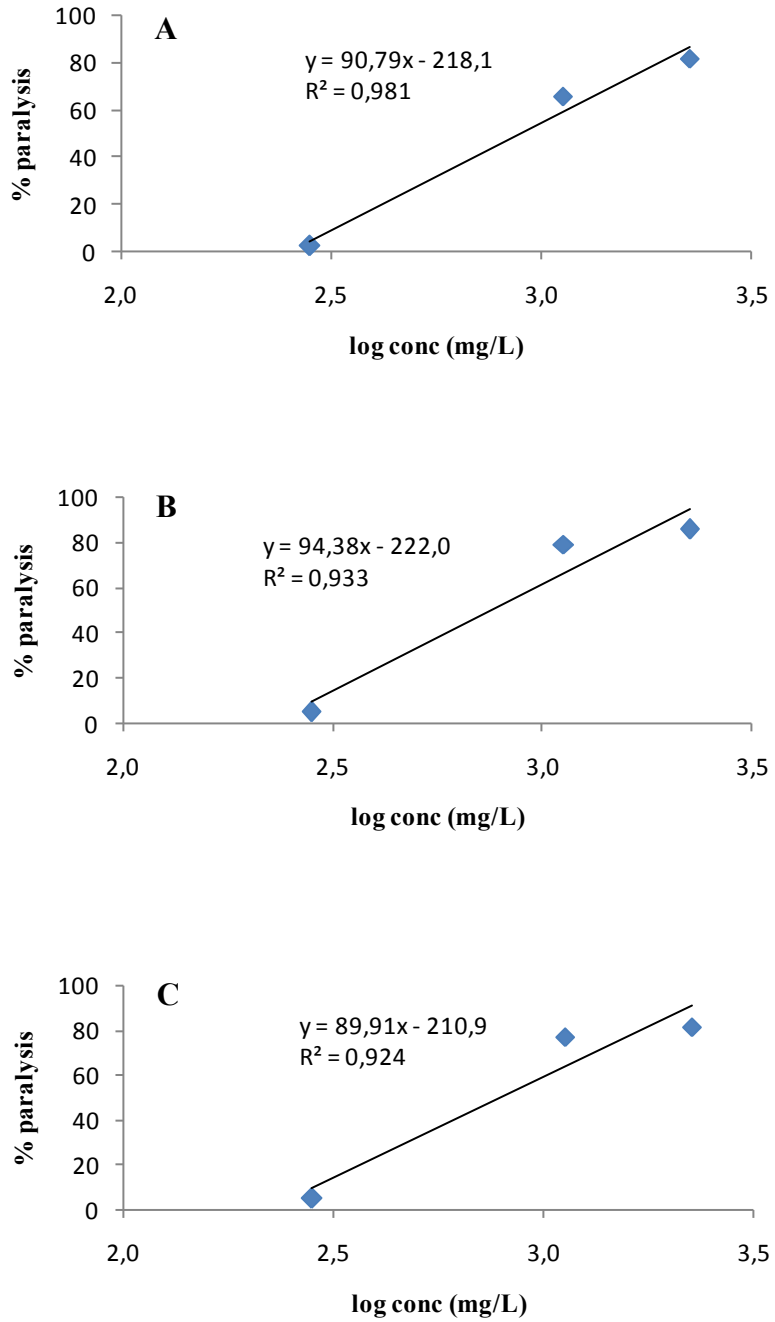


Figure 8. Relationship between the log concentration of 2-hydroxybenzaldehyde and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

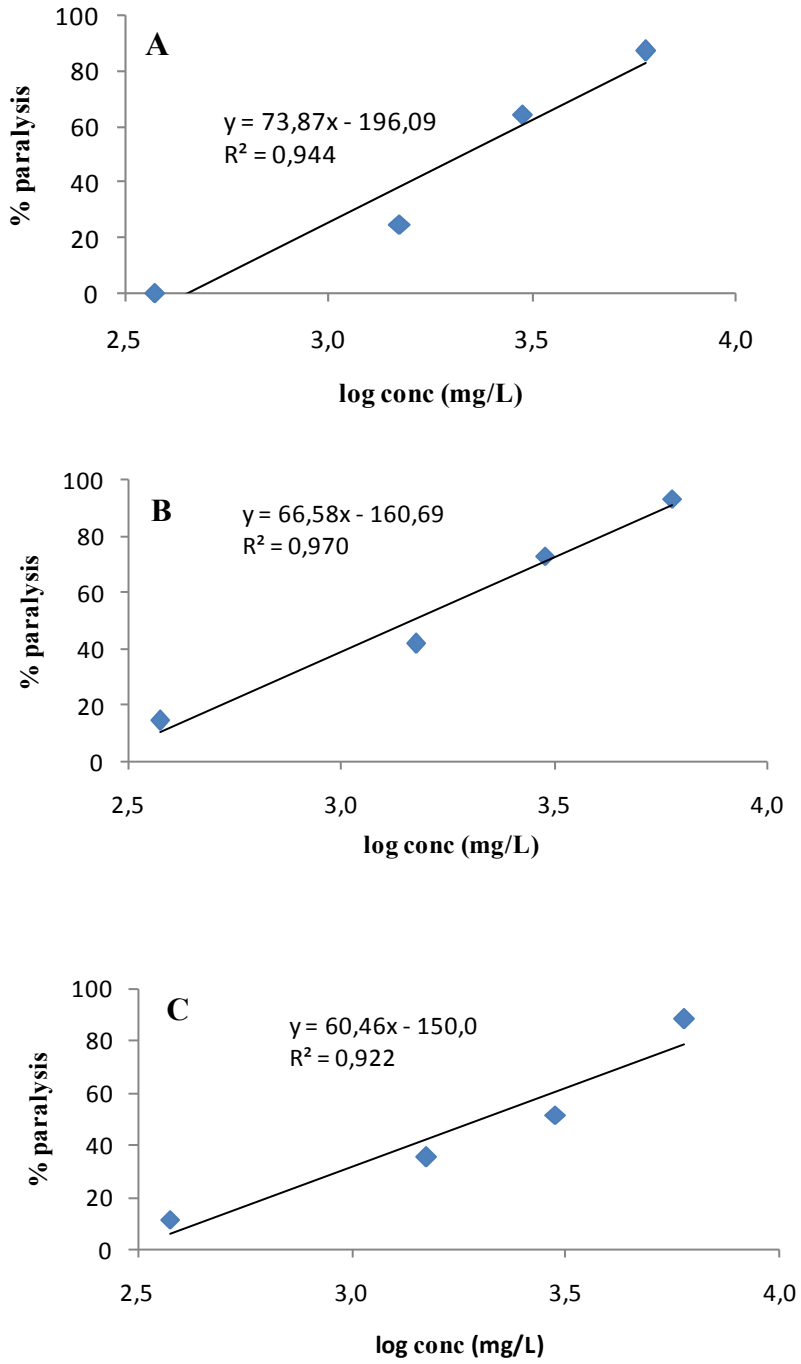


Figure 9.. Relationship between the log concentration of epigallocatechingallate and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

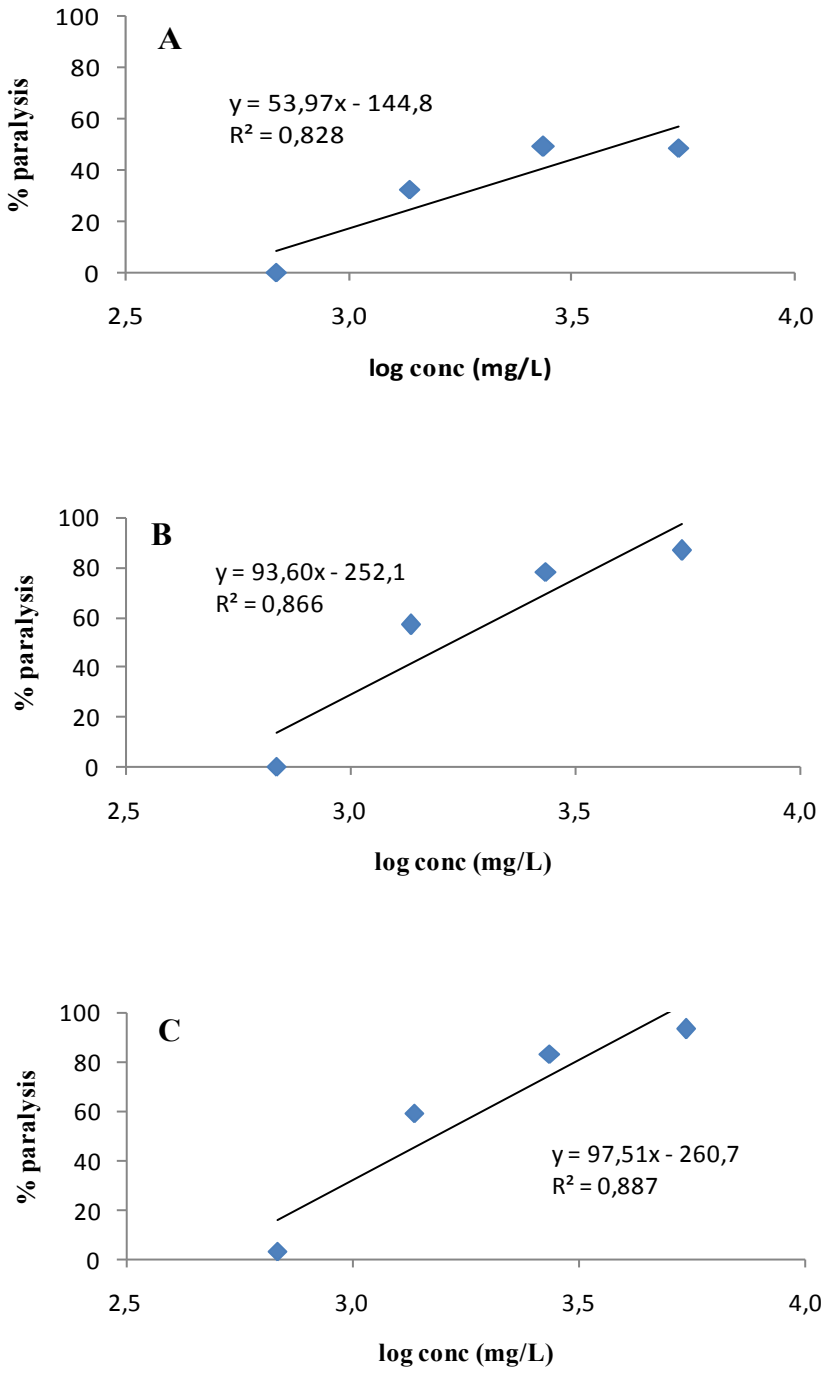
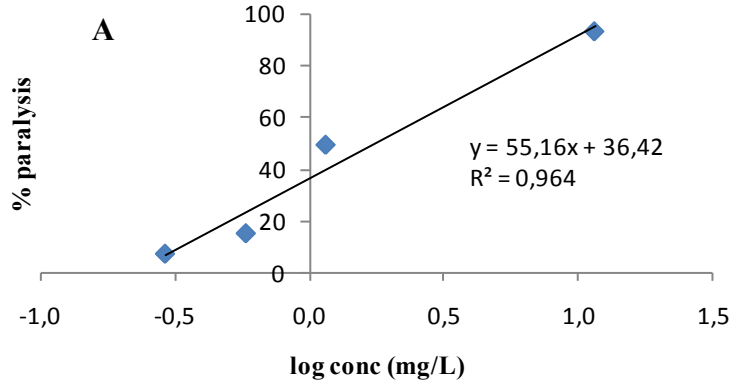
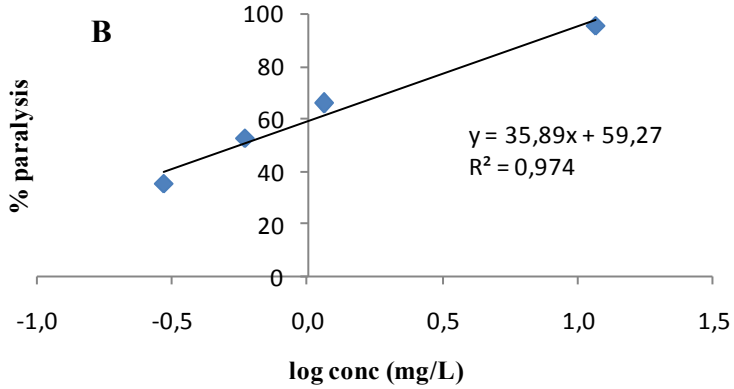


Figure 10. Relationship between the log concentration of levamisole hydrochloride and paralysis % of gastrointestinal nematodes at 1h (A), 24h (B) and 48h (C) after treatment.





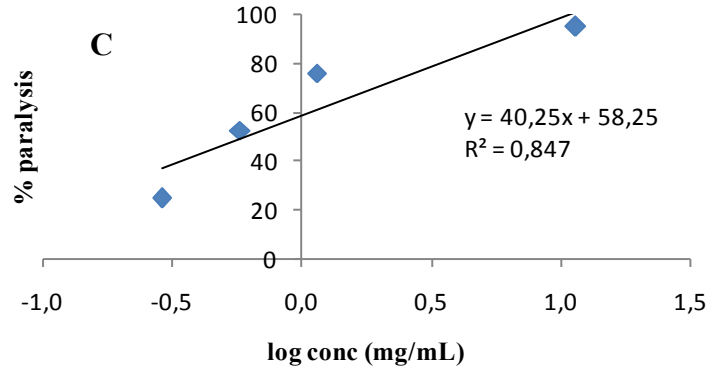


Figure 11. Relationship between the log concentration of furfural and paralysis % of lungworm L_1 nematodes at 1h (A), 24h (B) and 48h (C) after treatment.

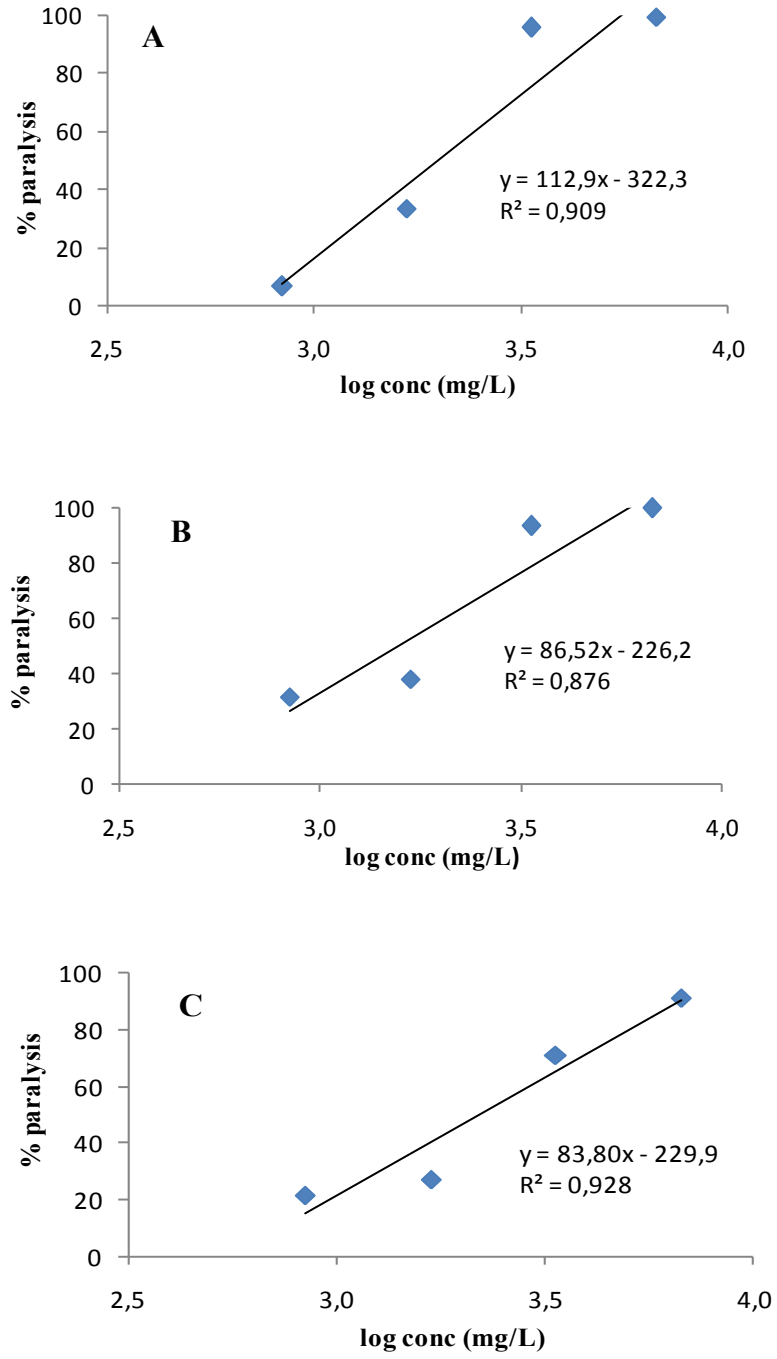
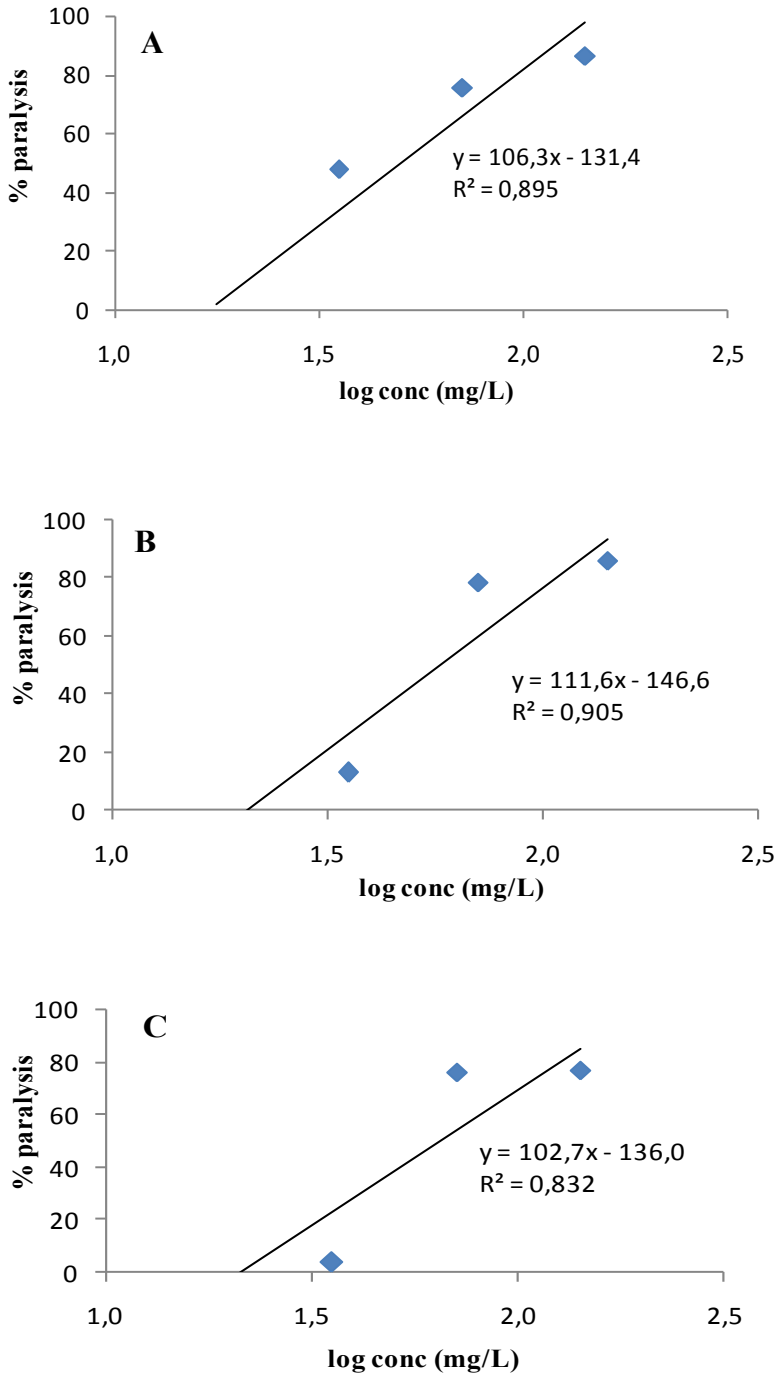


Figure 12. Relationship between the log concentration of 2-hydroxybenzaldehyde and paralysis % of lungworm L_1 nematodes at 1h (A), 24h (B) and 48h (C) after treatment.



CHAPTER 3

Effect of increasing doses of furfural and 2-hydroxybenzaldehyde in lactating dairy ewes. Consequences on diet utilization, health status and carry over into milk

Elisa Ortu

Effect of increasing doses of furfural and 2-hydroxybenzaldehyde in lactating dairy ewes. Consequences on diet utilization, health status and carry over into milk

Abstract. Gastrointestinal nematode resistance to conventional anthelmintic drugs is a serious and global issue that affect small ruminant herds. Plant extracts appear to be a valid alternatives to conventional anthelmintic drugs as source of secondary metabolites. In this study, compounds of botanical origin such as furfural and 2-hydroxybenzaldehyde were orally administered to Sarda dairy ewes. The aim of the work was to evaluate the effects of these compounds on Sarda dairy ewes, especially on feed intake, faecal and urine excretion and their potential carry over into milk. For this reason, residues were quantified by high performance liquid chromatography (HPLC-DAD) analysis. Moreover, after compounds administration, blood samples were collected for a toxicological screen. In faecal, urine and milk samples furfural, 2-hydroxybenzaldehyde and their metabolites were below the limit of determination. The absence of drug residues in milk samples during the four days after treatment suggest no carry over effects in milk. This information can be useful for further studies to evaluate the withdrawal period of these potential anthelmintic drugs. All blood parameters were within the normal range and no toxicological effects were observed in the ewes treated with furfural and 2-hydroxybenzaldehyde. In the future, we will investigate the *in vivo* anthelmintic activity, optimize the drug formulations and their mode of action.

1. Introduction

The frequent use of anthelmintic drugs to control gastrointestinal infestations in small ruminants is growing and is responsible of the development of drug resistance. There is an urgent need to discover and experience compounds with new mode of action. The optimal drugs should have a great anthelmintic effect and a good tolerability with low toxicity for the guest animals. In the last years, the attention has been focused towards of secondary metabolites of botanical origin. Furfural or 2-furaldehyde (F) is a heterocyclic aldehyde derived from a variety of agricultural byproducts such as corncobs, oat and wheat bran. It is a principal volatile component of *Melia azedarach* fruits, *Vanilla planifolia* beans and at a low concentration in *Ailanthus altissima* wood (Caboni et al., 2012; Ntalli et al., 2010; Pérez-Silva et al., 2006). Furthermore, it is used as solvent extraction in the petroleum refining industry, also as an intermediate in the synthesis of furan derivatives, as a fungicide and as a flavouring agent (Mc Killip and Sherman, 1980). The F is formed during thermal treatment of food by acid-catalyzed dehydration of carbohydrates containing hexose or pentose fragments, Maillard reaction or heat induced caramelization (Chheda et al., 2007; Kroh, 1994; Martins et al., 2000). This compounds has been identified in several foods and beverages, including bread, cocoa, milk products, cognac, tea, rum, port wine, coffee and juices (Kroes, inchem.org). Moreover, the oral LD_{50} for F was reported to be 100-127 mg/kg bw in rats (Sigma Aldrich, Milan Italy; Jenner *et al.,* 1964) and 333 mg/kg bw in mice (Boyland *et al.,* 1940). Several methods for the determination of F in different matrices are known. They include gas chromatography (GC-FID and GC/MS) for air and urine samples (Eller, 1994; Šedivec and Flek, 1978) and HPLC for determination in juices, distilled liquor and brandies (Lo Coco et al., 1994; Villalón Mir et al., 1992). Furthermore, Albalà- Hurtado et al. (1997) described an HPLC method for the determination of free and total furfurals including 5-hydroxymethylfurfural, furfural, furyl methyl ketone, and methylfurfural in milk. In humans, F is converted to furfuryl alcohol by enteric bacteria (Boopathyet al., 1993) and both compounds are rapidly absorbed from the gastrointestinal tract and totally excreted mainly in urine within 24 hours (Nomeir et al., 1992). After absorption, furfuryl alcohol is oxidized to F and further to 2-furoic acid (FA). The FA is then conjugated with glycine or converted to 2-

furanacryloyl-CoA and then to 2- furanacrylic acid and its glycine conjugate (Parkash and Caldwell, 1994) GC and HPLC methods have been used for the determination of FA in urine (Nutley, 1989; Tan et al., 2003). On the other hand, 2-hydroxybenzaldehyde or salicylaldehyde (2-HBA) is an aromatic aldehyde used as a precursor of other chemical compounds. It was identified as main component of buckwheat (*Fagopyrum esculentum*) aroma (Janeš and Kreft, 2008; Janeš et al., 2009), in the flowers of *Spirea ulmaria*, in the roots of *Crepis foetida* L. and in tobacco leaves. It is used in food as flavouring ingredient. Moreover, it has been identified in several food, such as potato, tomato, grape, mushrooms, pennyroyal oil (*Mentha pulegium*, L.) and in beverages including coffee, rum, beer and tea (Legacy tobacco documents library, University of San Francisco, California; Burdock, 1997). The oral LD_{50} for 2-HBA was reported to be 520 mg/kg bw in rats (Merck, Italy). Janeš et al. (2009) described a GC-MS method for the determination of buckwheat aroma included 2-HBA. Taking into consideration the *in vitro* nematicidal activity of furfural and 2-HBA against gastrointestinal nematodes (GIN), aim of this work was to evaluate their effect on health status of lactating Sarda dairy ewes, on diet utilization and their possible carry over into milk. Furthermore, the residual drugs in urine and faeces were investigated, considering also their metabolites and compounds with similar chemical structure such as 5-hydroxymethylfurfural (5-HMF), 2-furoic acid (FA) and salicylic acid (SA). In addition, the potential toxicological effects on the ewes were studied.

2. Material and methods

Chemicals

Standards of 5-HMF, F, FA, 2-HBA, SA, of purity greater than 98%, as well as acetonitrile, oxalic acid, boric acid, ethyl acetate, trichloroacetic acid (TCA) and ammonium formate were purchased from Sigma-Aldrich (Milano, Italy). Water used was purified on a Milli-Q apparatus. Acetonitrile used was of HPLC grade.

Experimental design

The experiment was carried out in 15 Sarda multiparous dairy ewes (aged between 3 and 7 years, weighing between 35.6 and 47.0 kg) at AGRIS farm located in Bonassai, Sassari, Italy. The experiment was carried out between June and July 2012 following the European Commission Council Directive that regulates the use of animals for experimental and other scientific purposes. Moreover, experimental procedure were approved by Comitato Indipendente di Bioetica per l'utilizzo di Animali a fini Sperimentali di Ateneo (C.I.B.A.S.A.) of the University of Sassary, Italy. The animals were acclimatized 7 days before the experiment and for 3 days were maintained indoors in individual metabolic cages. During the preliminary phase and the effective experiment, each animal received a daily diet consisting on dehydrated alfalfa (0.7 kg), alfalfa hay (1 kg), beet pulps (0.3 kg) and mix pelleted concentrate (0.6 kg). During the experiment, each ewe received individually appropriate mix of meal with individual buckets placed in the metabolic cages. During the morning milking, each ewe received the first portion of mix pelleted concentrate and when the milking time was finished, the buckets were taken out of the metabolic cage to be measure the residual of feeds. Later, ewes received dehydrated alfalfa and then during the evening milking time the second portion of mix pelleted concentrate was supplied. The mix was made available during the milking time and then the residual portion was weighed. On the other hand, alfalfa hay and beet pulps were made available until the next morning, when the residual portions were weighed. Furthermore, the ewes had water *ad libitum* through an automatic drinker. During the preliminary period, all animals were subjected to measurements of faecal worm egg counts (FWECs), milk yield and quality, body

weight (BW) and body condition score (BCS). Before the start of the experiment, animals did not receive treatment with anthelmintic product and animals were confirmed to be naturally infected by GIN. Three days before the start of the experiment, the animals were divided into three groups:

- group 1 (control): was a negative control (i.e. not treated);
- group 2 (furfural): ewes received, once a week, F in a single-dose rising for three weeks;
- group 3 (2-hydroxybenzaldehyde): ewes received, once a week, 2-HBA in a single-dose rising for three weeks.

Each group were statistically balanced according to BCS, BW, milk production and as a last step to FWECs. The sixth and seventh day of only experimental week consisted in a wash out time.

Drug formulation.

The F and 2-HBA doses were dissolved in 10 mL of distilled water and orally administered with a syringe. Animals of group 1 received only 20 mL of distilled water, while animals of group 2 and 3 received a single oral dose of F and 2-HBA, respectively. Before each drug administration, samples were vigorously shaken with a vortex apparatus to favourite the total dissolution of the active ingredient. After dosing, the test tube containing the drug was re-rinsed with 10 mL of distilled water and administered to the animal. During the three experimental weeks, animals received increasing doses of the same drug, consisting in a single oral dose for week. During the trial, the animals treated with the drugs were always the same. The dosage used for each drug is shown in Table 1.

Faecal and urine samples.

Freshly faecal samples were collected from the rectum of each animal in the morning before drug administration and at days 7, 14, 21 and 28 after treatment and kept at 4 °C until use. They were used to evaluate the parasitological effects of the aldehydes used. FWECs of gastrointestinal strongyles were performed according to a modified McMaster technique (Raynaud, 1970; Rossanigo and Gruner, 1991). Moreover, individual daily faecal and urine excretions were pooled and weighted at 0, 24, 48, 72, 96 hours after treatment. To prevent bacterial growth in the urine, individual animal samples were treated daily with 2g of boric acid each 500 mL of urine in accordance with Jöbstl et al. (2010). The amount of boric acid for single animal was calculated as average value of urine excreted in the preliminary period. Moreover, faecal and urine samples of each animal were mixed and an aliquot of 100 g of faeces and 50 mL of urine, respectively were collected daily from the total mass excreted. Samples were used for the detection and quantification of the possible residual drugs administered. Samples were stored at -80°C until use.

Blood samples.

Individual blood samples were collected weekly by jugular vein before the administration and 72 hours after drug administration. Test tubes with the anticoagulant EDTA were used for the complete blood count (CBC) analysis, while for the biochemical profile test tubes without anticoagulant were used. Samples were stored at 4°C and then were delivered to the laboratory of Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Italy) for immediate analysis.

Milk samples.

Ewes were milked before treatment and 1, 3, 6, 12, 24, 36, 48, 60, 72 and 84 hours after the weekly drug administration. For each milking the milk yield was recorded and a sample of milk was taken. The milk sample was stored at -80°C until use for chemical analysis of residual drugs. Moreover, fresh milk samples were taken twice a week and

analyzed for milk composition (fat, protein, lactose and urea content, and somatic cell counts) at AGRIS laboratory in Bonassai (Sassari, Italy).

Feed samples.

During the 7 days of each experimental period, residual intake of meal was weighed daily for each animal. Therefore, feed samples were taken once a week and stored at -20°C until chemical analysis.

Clinical observations.

During the experiment, ewes were carefully monitored by a veterinarian in order to detect any adverse reaction to the treatment.

Chemical Analysis

Faecal analysis.

Standard stock solutions of FA, F, 5-HMF and 2-HBA were prepared daily in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) and stored at -20°C until use. The analysis were performed on HPLC using an Agilent Technologies 1100 series (Waldbronn, Germany) equipped with a quaternary pump, autosampler, a degasser system and a diode array detector (DAD). A reverse phase column Varian Pursuit Xrs C18 (250 mm x 4.6 mm x 5µm) was employed and a guard column of the same material was used. The gradient program was as follows: starting from aqueous ammonium formate and acetonitrile (50:50 *v/v*) to aqueous ammonium formate and acetonitrile (35:65 *v/v*) in 8 min and then decreasing to 50% of aqueous ammonium formate in 7 min. The flow rate was 1.0 mL/min and the injection volume was 50 µL. The UV-DAD analysis was performed at a wavelength of 280 nm for F, 5-HMF and 254 nm for 2-HBA and FA. Samples for F, 5-HMF and FA determination were prepared dissolving 1g of faeces previously dried at 65°C for 5 days and powder, into 2 mL of 40 % (*w/v*) TCA in a sealed tube to prevent evaporation. Each sample was

mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 30 min. at 4000 rpm (ALC 4218 centrifuge. Milan, Italy). Later, 100 µL of the upper phase were dissolved into 900 µL of a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. On the other hand, samples for 2-HBA analysis were prepared dissolving 1g of faeces previously dried and powder into 2 mL of ethyl acetate in a sealed tube to prevent evaporation. Each sample was mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 25 min at 4000 rpm (ALC 4218 centrifuge. Milan, Italy). Later, 100 µL of the upper phase were dissolved into 900 µL of a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. An aliquot of 100 µL of F and 2-HBA at desired concentration was added to 1g of powder faeces and extracted using the method indicated before for faecal samples. Three replicates of each matrix were analyzed. The fortification level used were in the range of 0.1 and 15 mg/L for F and for 0.1 and 13 mg/L 2-HBA. The recovery were calculated by interpolation in calibration curves of matrix matched standards.

Urine analysis.

The analysis were performed on HPLC using an Agilent Technologies 1100 series (Waldbronn, Germany) equipped with a quaternary pump, autosampler, a degasser system and a diode array detector (DAD). A reverse phase column Polaris C18 (300 mm x 4.6 mm x 5µm) was employed and a guard column of the same material was used. The separation was carried out in gradient at room temperature using aqueous ammonium formate 10 mM and acetonitrile as mobile phases. The gradient program was as follows: starting from aqueous ammonium formate and acetonitrile (90:10 *v/v*) to aqueous ammonium formate and acetonitrile (50:50 *v/v*) in 22 min and then decreasing to 0% of aqueous ammonium formate in 3 min and maintained this concentration for 5 min. The flow rate was 1.0 mL/min and the injection volume was 100 µL. The UV-DAD analysis was performed at a wavelength of 280 nm for F, 5-HMF, 254 nm for 2-

HBA and 310 nm for SA. Standard stock solutions were prepared daily in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) and stored at -20°C until use. Urine samples for analysis were prepared dissolving 1 mL of urine into 1 mL of mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Samples were homogenized for a minute with a vortex apparatus, then filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis.

Blood analysis.

Hemogram parameters such as red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume of red blood cells (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH); red cell haemoglobin content (CH), haemoglobin distribution width (HDW) red cells distribution width (RDW), total platelets (PLT), mean platelet volume (MPV), and total white blood cells count values such as white blood cells (WBCB), neutrophils cells (NEUTS), lymphocytes cells (LYMPHS), monocytes cells (MONOS), eosinophils cells (EOS), large unstained cells (LUC) and basophils cells (BASOS) were analyzed by using a Laser Cyte Analyzer (IDEXX Laboratories, Milan, Italy). On the other hand, the biochemical parameters, such as albumin (ALB), alkaline phosphatase (ALP), total bilirubine (BT), creatinine (CRE), gamma glutamil transpeptidase (GGT), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), urea (UR) and total protein (PROT), were determined by a clinical analyzer spectrophotometric method (Dimension RXL Chemistry Analyzer, Dade Behring, Munich, Germany).

Milk analysis.

The analysis were performed on HPLC using an Agilent Technologies 1100 series (Waldbronn, Germany) equipped with a quaternary pump, autosampler, a degasser system and a diode array detector (DAD). A reverse phase column Varian Pursuit Xrs C18 (250 mm x 4.6 mm x 5µm) was employed and a guard column of the same material was used. The gradient program was as follows: starting from aqueous ammonium

formate and acetonitrile (50:50 *v/v*) to aqueous ammonium formate and acetonitrile (35:65 *v/v*) in 8 min and then decreasing to 50% of aqueous ammonium formate in 7 min. The flow rate was 1.0 mL/min and the injection volume was 50 µL. The UV- DAD analysis was performed at a wavelength of 280 nm for F, 5-HMF, 254 nm for FA, 2-HBA and 310 nm for SA. Standard stock solutions were prepared daily in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) and stored at -20°C until use. Samples for F, 5-HMF and FA determination were prepared dissolving 5g of milk into 1.5 mL of 0.15 M oxalic acid (freshly prepared) and 1 mL of 40 % (*w/v*) TCA in a sealed tube to prevent evaporation. Each sample was mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 15 min. at 3000 rpm (ALC 4218 centrifuge. Milan, Italy). The two phases were separated, 3 mL of 4 % (*w/v*) TCA were added to solid residue obtained and the sample was mixed for 10 minutes and centrifuged for 15 min. at 3000 rpm. TCA extract was transferred in a 25 mL volumetric flask and was filled the volume with 4 % (*w/v*) TCA. Later, samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. The sample preparation was in accordance to Albalà-Hurtado et al. (1997) with slight modifications. On the other hand, samples for 2-HBA and SA determination were prepared dissolving 2g of milk into 2 mL of ethyl acetate and a sealed tube to prevent evaporation. Each sample was mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 30 min at 4000 rpm (ALC 4218 centrifuge. Milan, Italy). Then, 100 µL of the upper phase were dissolved into 900 µL of a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. An aliquot of 100 µL of F and 2-HBA at desired concentration was added to 5g of milk and extracted using the method previously indicated for milk samples. Three replicates of each matrix were analyzed. The fortification level used were in the range of 0.1 and 15 mg/L for F and for 0.1 and 13 mg/L 2-HBA. The recoveries were calculated by interpolation in calibration curves of matrix matched standards. On the other hand, for milk fat, protein content and lactose determination a Milkoscan 4000, (Foss Electric, HillerØd, Denmark) was used. While, for milk somatic cell count (SCC) a Fossomatic 5000 was used (Foss Eletric, HillerØd, Denmark). Therefore, milk urea was analyzed with an automatic system (Chem spec 150 based on infrared reading, Bentley Instruments, Chaska, Minnesota, USA).

HPLC- method validation.

Method validation for urine, faeces and milk samples was carried out according to European Guideline for the bioanalytical method validation (EMEA, 2009). The HPLC-DAD method was validate for, sensitivity, linearity, precision, accuracy and recovery.

- *Sensitivity.* To evaluate sensitivity of the method, the detection limit (LOD) and quantification limit (LOQ) were estimate. The LOD was defined as the lowest concentration of compound which produced a pick with a signal noise ratio (S/N) values of ≥ 3. While the LOQ was defined as the lowest concentration of compound which produce a peak with an S/N ratio of ≥ 10. These limit values were determined by interpolating standard calibration curves prepared in solvent.

- *Linearity of calibration curves.* Adequate amounts of 5-HMF, F, FA, 2-HBA and SA were dissolved in 10 mL mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Stock solutions were diluted with the same mixture and injected in triplicate for the preparation of calibration curves. 5-HMF, F, FA, 2-HBA and SA were quantified in urine, milk and faeces by interpolation in calibration curves of matrix matched standards in the range of 0.1-15 mg/L. Samples were fortified at the desired level of 5-HMF, F, FA, 2-HBA and SA and directly injected for HPLC analysis after dilution with blank sample. Calibration curves were created by plotting the concentration of these compounds against the standard peak area following the standard addition method. The linearity of the method was demonstrated using blank sample spiked with the standards at concentration level of 0.1-15 mg/L for 5-HMF, F, FA, 2-HBA and SA, respectively.

- *Precision and accuracy*. The precision and accuracy of the method were evaluated by analyzing the samples of 5-HMF, F, FA, 2-HBA and SA in matrix at four different concentration levels. The intra-assay (within-day) repeatability was established performing six injections for each level of the standard in matrix on the same day. While the inter-assay (between-day) reproducibility was evaluated in six consecutive days.

Feed samples.

Feed samples were grounded using a 1 mm screen and analyzed for neutral detergent fiber (NDF) using an Ankom 220 fiberanalyzer (Ankom™ technology, Fairport, NY, USA) in accordance to Van Soest et al. (1991). Moreover, dry matter (DM) and ether extract were analyzed (AOAC, 1990a). The extract ether content was determined by the Soxtec System HT 1043 Extraction Unit (Tecator, Foss, Amersfoort, The Netherlands). Ashes (Ash) and crude protein (CP) content were determined following the AOAC (1990b). For CP determination, mineralization was carried out with a Tecator™ Digestion Unit 8 (FOSS Slangerupgade 69, DK-3400 Hilleroed, Denmark) and a Kjeltec™ 2200 Auto Distillation unit (FOSS Analytical 69, Slangerupgade DK-3400 Hilleroed Denmark). The data shown in the Table 2.

Statistical analysis

All data were statistically analysed using Minitab 16 software (Minitab Inc., State College, PA).

Faecal and urine data.

Faecal and urine data were analyzed by a general linear model ANOVA design: group and time effects were analyzed. The three weeks were analyzed separately, corresponding to the three increasing doses of F and 2-HBA. Moreover, for each week were considered four times corresponding to the data acquired 24, 48, 72 and 96 hours after treatment. Therefore, faecal and urine data before each weekly treatment were not considered because no differences were observed. The results of statistical analysis are shown in Table 3 and 4.

Blood data.

Blood serum biochemistry, hemogram, total and differential white blood cells count data were analyzed by a general linear model ANOVA design. The treatment effects were analyzed. For the statistical blood serum biochemistry analysis, the three weeks corresponding to the three different doses administered were considered separately, while the three groups were considered all in the same analysis. In the first week were detected significant differences between the data before each weekly treatment. For this reason, it was essential covariate the data corresponding to 72 hours after treatment with the data before treatment (Table 5). On the contrary, in the second and third week the data before each weekly treatment were not considered because were no significant differences between them and for this reason it was not essential covariate (Tables 6 and 7). For the statistical hemogram and, the total and differential white blood cells count analysis, was essential covariate data corresponding to 72 hours after treatment with the data before treatment of each week because were significant differences between them (Tables 8, 9, 10 and 11). The statistical analysis was carried out considering only the two last weeks of experiment because the data of the first period were not available.

Milk data.

Milk yield and composition data were analyzed by a general linear model ANOVA design: F and 2-HBA groups were analyzed separately with the aim of study only the differences of the single drug group respect to the control. The three weeks studied corresponding to the three increasing doses administered during the experiment. The week and the treatment effects were analyzed (Tables 12 and 13). Milk yield data were indicated as kg/day for each animal and were considered the milk production of the five days subsequent to treatment. On the other hand, the SCC data were reported as log10 cell/mL and for each ewe was considered a daily mean value. In the statistical analysis were not considered for each week, the data acquired the day of the drug treatment. It was possible to perform this selection after checking that there were no significant differences between groups for the data before each weekly treatment. Moreover, for fat, protein, lactose content and urea were considered the data related to the three days after drug administration.

Feed data.

Feed data were analyzed by a general linear model ANOVA design. The group and time effects were analyzed. Two classes of feed were processed in mix pelleted concentrate and forage. Forage class included dehydrated alfalfa, alfalfa hay and beet pulps. The three weeks were analyzed separately, and corresponding to the three increasing doses of F or 2-HBA administered. Moreover, for each week were considered four times corresponding to the data acquired 24, 48, 72 and 96 hours after treatment. Therefore, the data before each weekly treatment did not showed any significant differences. The statistical analysis are shown in Table 14 and 15.

Parasitological data.

Parasitological data were analyzed by a general linear model ANOVA design. The treatment and dose effects were analyzed. The three doses analysed, corresponded to the faecal samples collected at 72 hours after each weekly treatment. Therefore, the data before each weekly treatment were not considered because no significant differences were observed. The statistical analysis is shown in Table 16.

3. Results and Discussion

Drug formulation.

The F and 2-HBA formulations were prepared taking into account the water solubility and LD_{50} of the two compounds. F and 2-HBA have a partition-coefficient, known as logP, of 0.41 and 1.81, respectively. Therefore, F is more soluble in water than 2-HBA with a solubility of 83g/L at 20°C; while 2-HBA have a solubility equal to 14g/L at 20°C. Moreover, the oral LD_{50} in rats for F and 2-HBA were 100-127 mg/kg bw and 520 mg/kg bw, respectively. The evaluation of the LD_{50} was performed using rat data because there are no information in literature about oral acute toxicity in ruminants. Taking into account these parameters, the three increasing doses of F and 2-HBA prepared for the experimentation were the following: 1/80, 1/40 and 1/20 compared to oral LD_{50} in rats. Therefore, F and 2-HBA were tested in three consecutive weeks with

the following concentrations: 1.2, 2.5, 5.0 mg/kg bw for F and 6.5, 13.0, 26.0 mg/kg bw for 2-HBA. For the calculation of the individual doses, was considered for each group the mean of body weight.

Chemical composition

Faecal analysis.

Since F and 5-HMF showed a strong UV absorption at 280 nm (Figures 2 and 3) while FA and 2-HBA showed a maximum at 254 nm (Figures 4 and 5) and SA at 310 nm (Figure 6) we decided to develop and validate a reversed-phase HPLC method using photodiode array for measuring levels of these compounds in faecal samples. Taking into account that 5-HMF and F were unstable under light conditions, all samples were analyzed fresh. HPLC retention

times of FA, 5-HMF, F and 2-HBA were 2.2, 2.7, 3.8 and 6.3 minutes, respectively (Table 17 and Figure 7). Compounds of interest were not detected in faecal samples. These results suggest that probably the drugs were metabolized in other compounds or excreted through other ways.

Urine analysis.

As well as faecal samples, the UV-DAD analysis was performed at a wavelength of 280 nm for F and 5-HMF, 254 nm for 2-HBA. Moreover, the detection of SA occurred at 310 nm. HPLC retention times of 5-HMF, SA, F and 2-HBA were 6.1, 6.8, 8.5 and 19.4 min., respectively (Table 18). Urine samples did not require extraction steps but were prepared only for dilution and filtration. For this reason, recovery studies were not essential. All compounds analysed were not detected in urine samples. Boopathy et al (1993) indicated that F in human is converted to furfuryl alcohol by enteric bacteria, absorbed from the gastrointestinal tract and totally excreted mainly in urine within 24 hours (Nomeir et al., 1992). Moreover, furfuryl alcohol is oxidized to F and further to FA. The FA is then conjugated with glycine or converted to 2-furanacryloyl-CoA and then to 2- furanacrylic acid and its glycine conjugate (Parkash and Caldwell, 1994). F

and FA were searched but no the glycine conjugate and 2-furanacrylic acid. Probably, F and 2-HBA were transformed in other compounds by rumen microorganisms.

Milk analysis.

As well as faecal samples, the milk analysis were carried out using a Varian Pursuit Xrs C18 column and the same method, consequently the HPLC retention times (Table 17 and, Figure 7) and the UV wavelengths were the same. In addition, SA showed a retention time of 2.2 minutes and UV absorbance at the wavelength of 310 nm. Taking into account that FA and SA were analysed in different matrices their similar retention time did not pose an analytical challenge. FA, SA, 5-HMF, F and 2-HBA were not detected in milk samples, this fact led us to ruled out a carry-over phenomenon in milk. Generally, an essential peculiarity of all drugs used in ruminants producing milk for human consumption is minimize the withdrawal period. For example, for albendazole (Valbazen formulation) the withdrawal period in sheep corresponds to 4 days for milk (Ministero della Salute, 7 Giugno 2012) while monepantel can be used in breeding sheep including pregnant and lactating ewes but not in female sheep producing milk for human consumption (EMA, 2012). Minimizing this period, could be reduced the waste milk, thus limiting economical losses for farmers.

HPLC- method validation.

No interfering peaks were detected at the retention time of all compounds analyzed. Quantification limits (LOQ) calculated as signal noise ratio (S/N) of 10 were, 0.13 mg/L for 5-HMF, 0.20 mg/L for SA, 0.10 mg/L for F, FA, and 2-HBA, using a Varian Pursuit Xrs C18 column (Table 17). On the other hand, the LOQ calculated as signal noise ratio (S/N) of 10 were 0.10 mg/L for F, 5-HMF, SA and 2-HBA using a Polaris C18 column (Table 18). Detection limits (LOD) calculated as signal noise ratio (S/N) of 3 were 0.05 mg/L for FA, F, 5-HMF, 2-HBA and 0.10 mg/L for SA using a Varian Pursuit Xrs C18 column (Table 17). Using a Polaris C18 column, the LOD were 0.05 mg/mL for 5-HMF, SA, F and 2-HBA (Table 18). The linearity of the calibration curves for FA, 5-HMF, F, SA and 2-HBA in spiked samples is reported in Table 17 and 18. Calibration

curves consisting of concentration values were in the range of 0.10-15 mg/L for 2-HBA, F, S, FA and 5-HMF using a Varian Pursuit Xrs C18 column. Moreover, the mean correlation coefficients (R^2) of calibration curves were 0.999 for 5-HMF, F, 2-HBA, S and 0.992 for FA, respectively. On the other hand, the calibration curves consisting of concentration values were in the range of 0.10-15 mg/L for all compounds and the mean correlation coefficients (R^2) of calibration curves using a Polaris C18 column were 0.999 for 5-HMF, F, SA and 2-HBA. Recovery percent of F and 2-HBA on faecal samples were in the range of 74.2–97.3 and 85.1–93.9, respectively (Table 19). Instead, recovery percent of F and 2-HBA on milk samples were in the range of 88.6–95.4 and 90.8–110.1, respectively (Table 20). The proposed HPLC-DAD method, using to different columns, without the need of liquid-liquid extraction of chemical derivatization steps, can be considered fast and adequate to monitoring FA, 5-HMF, F, 2-HBA and S in faecal, urine and milk samples.

Statistical analysis

Faecal and urine data.

Urine and faecal production were statistically analyzed through a general linear model ANOVA design. Data related the period before each weekly treatment were eliminated because there were no significantly differences. The faecal production during the three weeks and in function on increasing doses of drugs did not present significant differences (Table 3). With regard to urine excretion, were observed significant differences in function on group (Table 4). In the second week, the urine production were significant different ($P \leq 0.05$) between F and 2-HBA groups, while the control group was similar to the other two groups. Urine production in F group was higher with a mean excretion values of 2.4 kg/day, than 2-HBA and control groups, but only statistically different by 2-HBA group with a mean excretion values of 1.5 kg/day. Moreover, in the third week, urine excretion in F group was significantly higher than 2HBS and control groups ($P \leq 0.01$) with mean excretion values of 2.5, 1.4, 1.5 kg/day, respectively. Instead, 2-HBA and control groups were no significantly different between them.

Blood data.

Blood serum biochemistry was analyzed evaluating three different weeks corresponding with the increasing doses of drugs used. Each week was statistically analyzed studying the differences through control, F and 2-HBA groups. For the first week (Table 5), corresponding to the low dose of drugs, it was essential covariate the data 72 hours after treatment in function of data acquired before to treatment, because existed significant differences through groups in the period previous to treatment. For ALP, BT, CRE, GGT, GPT and PROT were not observed significant differences. On the other hand, there were significant differences ($P \leq 0.05$) for ALB and GOT. The two parameters were within the normal range for all groups, but albumin was slightly higher in F group than in control and 2-HBA groups with a mean values of 2.8, 2.7, 2.7 g/dL, respectively. Moreover, GOT were higher in F and 2-HBA group than in control group with a mean values of 113.2, 117.0, 104.6 U/L, respectively. Furthermore, there were significant differences only between 2-HBA and control groups. Experimental studies in rats shown that F exerted hepatotoxicological effects and this information could be explain the GOT value higher in F group respect to the control group. On the other hand, for 2-HBA, is no known hepatological effects in experimental animals. In the second (Table 6) and in the third week (Table 7), corresponding to the medium and high dose of drugs administered, there were not significant differences between the three groups before weekly treatment. For this reason, these data were not considered in the statistical analysis. In both periods, for all parameters there were no significant differences. In addition, hemogram (Table 8 and 9) and, total and differential white blood cells count (Table 10 and 11) were statistically analyzed during the medium and high dose. The data relating to the low dose were no available. It was essential covariate the data corresponding to 72 hours after treatment in function of data before treatment. Concerning to hemogram parameters of the medium dose, there were significant differences in RBC, HGB, and HCT ($P \leq 0.05$), between F and 2-HBA groups. Instead, the control group did not show significant differences respect to F and 2-HBA groups . Moreover, for the three parameter F values were higher than the other two groups. In all cases, the parameters were within the normal range. On the other hand, for all parameters analyzed, including hemogram and, total and differential white blood cells count in the second and third group were not observed significant differences.

Milk data.

A statistical analysis was carried to study the milk yield and chemical composition of milk. It were considered three levels corresponding to the three increasing doses administrated during the experiment. Moreover, were analyzed separately two treatments: control against F (Table 12) group and control against 2-HBA group (Table 13). With regard to F, there were no significant differences for milk yield, somatic cell counts and lactose content. The milk production during the three weeks of the experiment decreased in accordance with the lactation curve but were not observed significant differences. On the other hand, were detected significant differences ($P \leq 0.01$) between groups, for fat content, protein content and urea. Furthermore, between weeks and treatments data there were not significant differences ($P > 0.05$). The differences detected in fat and protein content between control and F groups could be caused by a reduced production on milk in F group respect to control group. The milk production between groups was not significant but F group produced on average 110 g less than control group; consequently this reduction could be influenced the fat and protein content. With regard to urea levels in milk, the control group showed a mean value significantly higher (46.7 mg/dL) than the F group (40.2 mg/dL).Milk urea is a constituent part of the non-protein nitrogen normally found in milk and it is correlated with the urea concentration in blood. Urea levels in blood are influenced by different parameters such as protein intake, energy intake, and urinary excretion. Since urea is passed out of the body in the urine, increasing water intake, which may increase urinary production and decrease blood urea concentration. In this experiment no significant differences were observed between groups for blood urea levels. Statistical differences were observed for milk urea concentration between F and control groups. Concerning urine excretion, in the second and third weeks in the F group the excretion was greater than in 2-HBA and control groups. The lower levels of milk urea in the F group were present from the first period and no was a consequence of a major urine excretion because blood urea values in F group were similar to the other two groups. On the other hand, the statistical analysis between control and 2-HBA groups did not showed significant differences in somatic cell counts, protein content, lactose content and urea concentrations. Moreover, the milk production during the three weeks of experiment decreased in accordance with the lactation curve but there were not significant

differences. On the contrary, in milk yield and fat content were detected significant differences ($P \leq 0.01$) between treatments. In the 2-HBA group, a reduction on milk production can influence the milk fat content.

Feed data.

The residues of forage consisting on dehydrated alfalfa, alfalfa hay and beet pulps were monitored and weighted daily during the experimental trial to verify if the drugs administrated could influence the feed ingestion. Moreover, ewes received a mix of pelleted concentrate and the residues were monitored and weighted daily. It was conducted a general linear ANOVA design for daily intake of forages and mix pelleted concentrate ingested (Tables 14 and 15). It was no essential applied covariance because there were no significant differences through groups during the period before treatment. Concerning the forage ingestion, during the three weeks of treatment, were not observed significant differences in function on time. On the other hand, in the first week, corresponding to the low dose of drugs administrated, there were significant differences through groups ($P \leq 0.01$). Especially, the F group showed a daily intake less than the control and 2-HBA groups with a mean values of 1.7, 1.9, 1.8 kg/day, respectively. Concerning the daily intake of mix pelleted concentrate, in the first week F group were less significant (0.5 kg/day) if compared with the control and 2-HBA groups (both 0.6 kg/day) with a P value ≤ 0.01. Moreover, in the third week, corresponding to the high dose of drugs administered, the daily feed intake 48 hours after treatment was less significant (0.4 kg/day) respect to the daily intake 24, 72 and 96 hours after treatment (0.6 kg/day) with a P value ≤ 0.01.

Parasitological data.

FWECs data were statistically analyzed by a general linear model ANOVA design with the aim of evaluating the parasitological effects of the treated groups. There were no significant differences through control and treated groups (Table 16) but different trends can be observed through groups (Figure 1). The three groups at the beginning of the treatment have indicatively similar epg mean values. In the control group, the epg mean

increased throughout the all trial while in 2-HBA group epg mean increased after administration of the low and medium dose but after the high dose appear to stabilize. Anyway, the 2-HBA group at the end of experiment had an increased epg mean but showed a different trend respect to control group.

On the other hand, in the F group the egp mean increased at low and the medium dose administrated while decreased at the high dose. Figure 1 showed a clearly different trend of group treated with F and 2-HBA respect to the control group.

Clinical observations.

Ewes during the experiment were kept under control to monitor possible negative and toxicological effects caused by administered drugs. Health status of the animals were monitored with the help of a veterinarian, before drug treatment and 72 hours after treatment. No animal showed toxicological symptoms.

4. Conclusions

Fast, reproducible and robust HPLC methods were developed to evaluate F, 2-HBA, FA, 5-HMF and SA concentration in faecal, urine and milk samples. F, 2-HBA and their metabolites were no detected in faecal, urine and milk samples. Thus suggesting no carry over effects in the milk, while this information may be useful for further studies to evaluate the withdrawal period of these drugs. Taking into consideration that 5-HMF is a heterocyclic aldehyde considered a potential carcinogenic it was comforting not detected it in milk. F and 2-HBA can be transformed in the rumen or in the liver to different metabolites not analysed in this work. In the future, could be interesting the use of a formulation protecting the drugs by the rumen while retaining their drug action in the abomasum, large and small intestine where gastrointestinal nematodes are resident. Concerning blood data significant differences through groups were observed but parameters were within the normal range. The mean objective of this experimental trial was to evaluate the toxicological effects of F and 2-HBA and their possible carry over into milk. Anthelmintic effects was studied partially, giving anyway comforting

results. This study showed that the administration of F and 2-HBA to ewes did not showed toxicological effects and for this reason should be interesting investigate further their potentiality as anthelmintics.

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6. References

Albalá-Hurtado S., Veciana-Nogués M. T., Izquierdo-Pulido M., Vidal-Carou M. C. 1997. Determination of Free and Total Furfural Compounds in Infant Milk Formulas by High-Performance Liquid Chromatography. *J. Agric. Food Chem.*, 45, 2128–2133.

Association of Official Analytical Chemists, 1990a. Procedure 920.29 in *Official Methods of Analysis*. 15th ed. AOAC, Arlington, VA.

Association of Official Analytical Chemists, 1990b. Procedure 984.13 in *Official Methods of Analysis*. 15th ed. AOAC, Arlington, VA.

Boyland, E. 1940. Experiments on the chemotherapy of cancer. Further experiments with aldehydes and their derivatives. *Biochem. J.,* 34, 1196-1201.

Boopathy, R., Bokang, H., Daniels, L., 1993. Biotransformation of furfural and 5-hydroxymethylfurfural by enteric bacteria. *J. Ind. Microbiol.* 11,147-150.

Burdock G. A. 1997. Encyclopedia of Food and Color Additives. Volume 3, pag 2485. CRC Press, Inc.

Caboni P., G. Ntalli N., Aissani N., Cavoski I., Angioni A. 2012. Nematicidal activity of (E,E)-2,4-Decadienal and (E)-2-Decenal from *Ailanthus altissima* against *Meloidogyne javanica*. *J. Agr. Food Chem.*, 60, 1146−1151.

Chheda J. N, Leshkova Y. R., Dumesic J, A. 2007. Production of 5-hydroxymethylfurfural and furfural by dehydration of biomass-derived mono- and poly-saccharides. *Green Chem.*, 9, 342-350.

Eller, P. M. 1994. NIOSH Manual of Analytical Methods, 4th Ed., Vol. 1 (DHHS (NIOSH) Publ.No. 94-1 13), Washington DC, US Government Printing Office, Methods 2529,2539.

European Medicines Agency, 2012. European public MRL assessment report (EPMAR). Monepantel (caprine and ovine species). EMA/CVMP/165324/2008.

Kroh L. W. 1994. Caramelisation in food and beverages. *Food Chemistry*, 51, 373–379.

Janeš, D.; Kreft, S. 2008. Salicylaldehyde is a characteristic aroma component of buckwheat groats. *Food Chem.*, 109, 293–298.

Janeš D, Kantar D., Kreft S, Prosen H. 2009. Identification of buckwheat (*Fagopyrum esculentum* Moench) aroma compounds with GC–MS. *Food Chem.*, 112, 120–124.

Jenner P. M., Hagan E. C., Taylor J. M., Cook E. L., Fitzhugh O. G. 1964. Food flavourings and compounds of related structure. I. Acute oral toxicity. *Food and Cosmet. Toxicol.*, 2, 327–343.

Jöbstl D., Husøy T., Alexander J., Bjellaas T., Leitner E., Murkovic M. 2010. Analysis of 5-hydroxymethyl-2-furoic acid (HMFA) the main metabolite of alimentary 5-hydroxymethyl-2-furfural (HMF) with HPLC and GC in urine. *Food Chem.*, 123, 814–818.

Lo Coco F., Valentini, C., Novelli V., Ceccon L. 1994. High performance liquid chromatographic determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in processes citrus juices. *J. of Liquid Chromatogr*. 17, 603-617.

Martins S. I. F. S, Jongen W. M. F, Van Boekel M. A. J. S. 2000. A review of Maillard reaction in food and implications to kinetic modeling. *Trends Food Sci. Tech.*, 11, 364–373.

McKillip W J, Sherman, E. 1980. Furan derivatives. ln: Mark, H. F., Othmer, D. F., Overberger, C. G., Seaborg, G. T., Grayson, M., eds, Kirk-Othmer *Encyclopedia Of Chemical Technology*, 3rd Ed., Vol. II. John Wiley & Sons, New York. pp. 501-510.

Ministero della Salute, GU n.131 del 7-6-2012. Modificazione dell'autorizzazione all'immissione in commercio del medicinale per uso veterinario «Valbazen».

Nomeir A. A., Siveria D. M., Mc Comish M. F., Chadwick M. 1992. Comparative metabolism and disposition of furfural and furfuryl alcohol in rats. *Drug Metab. Disposit.* 20, 198-204.

Ntalli N. G., Vargiu S., Menkissogluspiroudi U., Caboni P. 2010. Nematicidal Carboxylic Acids and Aldehydes from *Melia azedarach* Fruits. *J. Agr. Food Chem.*, 58, 11390–11394.

Nutley B. P. 1989. An analytical method for the biological monitoring of furfural exposure. Health and Safety Executive Internal Report IR/L/OT/89/1.

Parkash M. K., Caldwell J. 1994. Metabolism and excretion of [14C]-furfural in the rat and the mouse. *Food Chem. Toxicol.* 32,887-895.

Pérez-Silva A, Odoux E., Brat P., Ribeyre F., Rodriguez-Jimenes G, Robles-Olvera V., García-Alvarado M. A., Günata Z. 2006. GC–MS and GC–olfactometry analysis of aroma compounds in a representative organic aroma extract from cured vanilla (*Vanilla planifolia* G. Jackson) beans. *Food Chem.*, 99, 728–735.

Raynaud J. P. 1970. Etude de l'efficacitè d'une technique de coproscopie quantitative pour le diagnostic de routine et le controle des infestations parasitaires des bovins, ovins, èquins et porcins. *Ann. Parasitol.*, 45, 321-342.

Rossanigo C. E., Gruner L. (1991). Accuracy of two methods for counting eggs of sheep nematode parasites. *Vet. Parasitol.*, 39,115-121.

Šedivec V., Flek J. 1978. Biologic monitoring of persons exposed to furfural vapors. *Int. Arch. occup. environ*. *Health*, 42, 41-49.

Tan Z. B., Tonks C. E., O'Donnell G. E, Geyer R. 2003. An Improved HPLC Analysis of the Metabolite Furoic Acid in the Urine of Workers Occupationally Exposed to Furfural. *Journal of Anal. Toxicol.*, 27, 43-46.

Van Soest P. J., Robertson J. B., Lewis B. A. 1991. Methods for dietary fiber, neutral detergent fiber and non starch polysaccharides in relation to animal nutrition. *J. Dairy. Sci.,* 74, 3583–3597.

Villalón Mir M., Quesada Granados J., López Garcia de la Serrana H., López Martinez M. C. 1992. High performance liquid chromatography determination of furanic compounds in commercial brandies and caramels. *J. Liq. Chromatogr.*, 15, 513-524.

http://www.inchem.org/documents/jecfa/jecmono/v042je03.h

https://legacy.library.ucsf.edu/tid/kli01d00/pdf

Table 1. Draft of the treatment sequence of dosages applied during the experiment.

group/treatment	Ewes (n.)	Preliminary	week 1	week 2	week 3
Control	5	0	0	0	0
Furfural, mg/ kg of BW	5	0	1.2	2.5	5.0
2-HBA, mg/ kg of BW	5	0	6.5	13.0	26.0

Table 2. Chemical composition of the feeds used in the experiment.

	Chemical composition				
	DM	**Ash**	**CP**	**EE**	**NDF**
	% as fed	**% DM**	**% DM**	**% DM**	**% DM**
Dehydrated alfalfa	88.62	9.26	19.17	2.56	42.53
Alfalfa hay	85.35	14.21	17.02	1.38	52.71
Beet pulps	88.81	8.49	10.43	0.69	48.94
Mix pelleted concentrate	88.38	11.95	16.07	2.81	38.02

DM= dry matter; Ash= ashes; CP= crude protein; EE=ether extract; NDF=neutral detergent fiber.

Table 3. Means of individual faecal production (kg/day**)** of Sarda dairy ewes during furfural and 2-hydroxybenzaldehyde treatments.

	Time[a]				Group			SEM	P-value[c]	
	24h	**48h**	**72h**	**96h**	**Control**	**F**	**2-HBA**		**time**	**group**
week1[b]	2.7	2.8	3.0	2.8	2.9	2.8	2.8	0.20	ns	ns
week 2	2.9	3.0	3.0	2.8	3.0	2.9	2.9	0.18	ns	ns
week 3	2.9	2.9	3.1	3.2	3.3	2.8	2.9	0.55	ns	ns

ns = not significant with P>0.05;
[a] time: 24h = 24 hours after treatment of F or 2-HBA; 48h = 48 hours after treatment of F or 2-HBA; 72h = 72 hours after treatment of F or 2-HBA; 96h = 96 hours after treatment of F or 2-HBA;
[b] week1: low dose; week2: medium dose; week3: high dose of F or 2-HBA;
[c] the interaction time-group was not significant.

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Table 4. Means of daily urine production (kg/head) of Sarda dairy ewes during the 3 weeks of increasing doses of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) administered.

	Time[a]				Group			SEM	P-value[c]	
	24h	**48h**	**72h**	**96h**	**Control**	**F**	**2-HBA**		**time**	**group**
week1[b]	1.4	1.3	1.4	1.5	1.5	1.6	1.2	0.17	ns	ns
week 2	1.8	1.7	1.7	2.2	1.7[ab]	2.4[a]	1.5[b]	0.59	ns	*
week 3	1.8	1.6	1.7	2.1	1.5[b]	2.5[a]	1.4[b]	0.49	ns	**

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

[a] time: 24h = 24 hours after treatment of F or 2-HBA; 48h = 48 hours after treatment of F or 2-HBA; 72h = 72 hours after treatment of F or 2-HBA; 96h = 96 hours after treatment of F or 2-HBA;

[b] week1: low dose; week2: medium dose; week3: high dose of F or 2-HBA;

[c] the interaction time- group was not significant.

Table 5. Blood serum biochemistry of Sarda dairy ewes during the first week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
ALB, g/dL	2.7[a]	2.8[b]	2.7[a]	0.06	*
ALP, U/L	94.5	94.1	87.0	12.7	ns
BT, mg/dL	0.3	0.3	0.3	0.04	ns
CRE, mg/dL	0.4	0.4	0.4	0.07	ns
GGT, U/L	84.9	90.6	90.7	3.9	ns
GOT, U/L	104.6[b]	113.2[ab]	117.0[a]	6.1	*
GPT, U/L	25.6	24.5	32.0	5.2	ns
PROT, g/dL	7.0	7.2	7.3	0.22	ns
UREA, mg/dL	63.8	63.9	66.2	5.9	ns

Different letters mean significant differences;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
Dose 1 = 72 hours after treatment with the low dose of F or 2-HBA;
ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gamma glutamil transpeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT= Glutamic Pyruvic Transaminase, PROT = Total Protein.

Table 6. Blood serum biochemistry of Sarda dairy ewes during the second week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
ALB, g/dL	2.7	2.8	2.8	0.10	ns
ALP, U/L	116.6	69.4	101.6	5.8	ns
BT, mg/dL	0.3	0.3	0.3	0.04	ns
CRE, mg/dL	0.5	0.5	0.5	0.06	ns
GGT, U/L	85.8	93.0	79.8	12.6	ns
GOT, U/L	101.0	121.0	123.6	20.1	ns
GPT, U/L	26.4	29.2	27.2	3.6	ns
PROT, g/dL	6.9	7.3	6.9	0.59	ns
UREA, mg/dL	70.8	63.6	68.8	7.9	ns

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
Dose 2 = 72 hours after treatment with the medium dose of F or 2-HBA;
ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gamma glutamil transpeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT= Glutamic Pyruvic Transaminase, PROT = Total Protein.

Table 7. Blood serum biochemistry of Sarda dairy ewes during the third week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
ALB, g/dL	2.7	2.7	2.7	0.10	ns
ALP, U/L	113.0	70.8	78.2	54.8	ns
BT, mg/dL	0.3	0.2	0.3	0.04	ns
CRE, mg/dL	0.4	0.5	0.4	0.04	ns
GGT, U/L	80.8	88.0	76.6	12.2	ns
GOT, U/L	102.6	125.6	133.8	20.2	ns
GPT, U/L	26.0	25.2	28.6	4.6	ns
PROT, g/dL	6.9	7.3	7.0	0.52	ns
UREA, mg/dL	72.0	60.8	67.6	9.3	ns

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

Dose 3 = 72 hours after treatment with the high dose of F or 2-HBA;

ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gamma glutamil transpeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT= Glutamic Pyruvic Transaminase, PROT = Total Protein.

Table 8. Hemogram of Sarda dairy ewes during the second week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
RBC, x 10^6 cells/µL	8.9^{ab}	9.3^{b}	8.3^{a}	0.49	*
HGB, g/dL	10.1^{ab}	10.5^{b}	8.8^{a}	0.77	*
HCT, %	29.0^{ab}	29.9^{b}	26.5^{a}	1.8	*
MCV, fl	32.2	32.5	32.4	0.36	ns
MCH, pg	11.2	11.5	10.7	0.68	ns
MCHC, g/dL	35.0	35.6	32.6	2.1	ns
CH, pg	10.1	10.2	10.2	0.09	ns
RDW, %	18.1	18.4	18.2	0.36	ns
HDW g/dL	2.3	2.3	2.3	0.04	ns
PLT, x 10^3 cells/µL	779.9	834.2	792.9	83.8	ns
MPV, fl	9.6	8.6	9.3	1.0	ns

Different letters mean significant differences; ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
Dose 2 = 72 hours after treatment with the medium dose of F or 2-HBA;
RBC = Red Blood Cells, HGB = Haemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volume of Red Blood Cells, MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration, CH= Red Cell Haemoglobin Content; RDW=; Red Cell Distribution Width; HDW=Haemoglobin Distribution Width; PLT= Total Platelets; MPV= Mean Platelet Volume.

Table 9. Hemogram of Sarda dairy ewes during the third week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
RBC, x 10^6 cells/µL	8.7	9.0	9.0	0.60	ns
HGB, g/dL	9.9	10.1	10.3	0.59	ns
HCT, %	28.5	28.8	29.0	1.93	ns
MCV, fl	32.8	31.8	32.3	1.58	ns
MCH, pg	11.3	11.2	11.5	0.73	ns
MCHC, g/dL	34.6	35.2	35.6	1.60	ns
CH, pg	10.5	10.3	10.5	0.44	ns
RDW, %	18.4	18.5	18.9	1.06	ns
HDW g/dL	2.4	2.4	2.5	0.10	ns
PLT, x 10^3 cells/µL	753.3	766.4	755.3	55.14	ns
MPV, fl	8.2	9.1	9.8	1.17	ns

Dose3 = 72 hours after treatment with the high dose of furfural or 2-hydroxybenzaldehyde;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
RBC = Red Blood Cells, HGB = Haemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volume of Red Blood Cells, MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration, CH= Red Cell Haemoglobin Content; RDW=; Red Cell Distribution Width; HDW=Haemoglobin Distribution Width; PLT= Total Platelets; MPV= Mean Platelet Volume.

Table 10. Total and differential white blood cells count of Sarda dairy ewes during the second week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
WBCB, x 10^3 cells/µL	7.6	6.8	7.2	0.78	ns
NEUTS, x 10^3 cells/µL	3.1	2.4	2.8	0.47	ns
LYMPHS, x 10^3 cells/µL	3.9	3.7	4.0	0.48	ns
MONOS, x 10^3 cells/µL	0.1	0.1	0.1	0.05	ns
EOS, x 10^3 cells/µL	0.4	0.4	0.3	0.08	ns
BASOS, x 10^3 cells/µL	0.1	0.1	0.1	0.02	ns
LUC, x 10^3 cells/µL	0.1	0.1	0.1	0.02	ns

Dose 2 = 72 hours after treatment of medium dose of F or 2-HBA;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
WBCB = White Blood Cells, NEUTS = Neutrophil Cells, LYMPHS = Lymphocytes Cells, MONOS = Monocytes Cells, EOS = Eosinophils Cells, Basos = Basophils Cells; LUC = Large Unstained Cells.

Table 11. Total and differential white blood cells count of Sarda dairy ewes during the third week of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Treatment			SEM	P-value
Parameter	**Control**	**F**	**2-HBA**		
WBCB, x 10^3 cells/µL	7.2	7.5	8.4	1.12	ns
NEUTS, x 10^3 cells/µL	3.1	2.4	2.8	0.91	ns
LYMPHS, x 10^3 cells/µL	3.6	3.6	3.9	0.58	ns
MONOS, x 10^3 cells/µL	0.1	0.2	0.3	0.17	ns
EOS, x 10^3 cells/µL	0.4	0.5	0.5	0.16	ns
BASOS, x 10^3 cells/µL	0.1	0.1	0.1	0.02	ns
LUC, x 10^3 cells/µL	0.1	0.1	0.1	0.02	ns

Dose 3 = 72 hours after treatment with the high dose of F or 2-HBA;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
WBCB = White Blood Cells, NEUTS = Neutrophil Cells, LYMPHS = Lymphocytes Cells, MONOS = Monocytes Cells, EOS = Eosinophils Cells, Basos = Basophils Cells; LUC = Large Unstained Cells.

Table 12. Effects on milk yield and chemical composition of Sarda dairy ewes during furfural (F) treatments.

	week[a]			treatment		SEM	P-value[b]	
	week1	**week2**	**week3**	**Control**	**F**		**week**	**treatment**
Milk yield, kg/day	1.4	1.4	1.3	1.4	1.3	0.06	ns	ns
SCC, log_{10} cell/mL	5.2	5.1	5.1	5.2	5.1	0.34	ns	ns
Fat,%	6.2	6.1	6.0	5.9	6.3	0.94	ns	**
Protein,%	5.9	5.8	5.8	5.7	6.0	0.51	ns	**
Lactose, %	4.8	4.7	4.7	4.7	4.8	0.45	ns	ns
Urea, mg/dL	44.2	43.1	43.0	46.7	40.2	6.58	ns	**

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

[a] week1 = Low dose of F (1.2 mg/kg); week2 = Medium dose of F (2.5mg/kg); week3 = High dose of F (5.0mg/kg);

[b] week x treatment interaction not significant.

Table 13. Effect on milk yield and chemical composition of Sarda dairy ewes during 2-hydroxybenzaldehyde (2-HBA) treatments.

	week[a]				treatment		SEM	P-value[b]	
	week1	week2	week3		Control	F		week	treatment
Milk yield, kg/day	1.4	1.4	1.3	1.4	1.3	0.06	ns	**	1.4
SCC, log_{10} cell/mL	5.4	5.3	5.3	5.2	5.4	0.32	ns	ns	5.4
Fat,%	6.3	6.2	6.1	5.9	6.5	0.85	ns	**	6.3
Protein,%	5.8	5.7	5.7	5.7	5.7	0.38	ns	ns	5.8
Lactose, %	4.7	4.7	4.7	4.7	4.7	0.13	ns	ns	4.7
Urea, mg/dL	47.7	46.9	46.4	46.7	47.4	5.19	ns	ns	47.7

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

[a] week1 = Low dose of 2-HBA (6.5 mg/kg); week2 = Medium dose of 2-HBA (13 mg/kg); week3 = High dose of 2-HBA (26 mg/kg);

[b] week x treatment interaction not significant.

Table 14. Means of daily intake of forage of Sarda dairy ewes during the 3 weeks of increasing doses of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) administered.

	Time[a]				Group			SEM	P-value[c]	
	24h	**48h**	**72h**	**96h**	**Control**	**F**	**2-HBA**		**time**	**group**
week1[b]	1.8	1.8	1.8	1.8	1.9[a]	1.7[b]	1.8[ab]	0.03	ns	**
week 2	1.8	1.8	1.8	1.8	1.8	1.7	1.8	0.08	ns	ns
week 3	1.8	1.8	1.8	1.7	1.8	1.7	1.8	0.17	ns	ns

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

[a] time: 24h = 24 hours after treatment of F or 2-HBA; 48h = 48 hours after treatment of F or 2-HBA; 72h = 72 hours after treatment of F or 2-HBA; 96h = 96 hours after treatment of F or 2-HBA;

[b] week1: low dose; week2: medium dose; week3: high dose of F or 2-HBA;

[c] the interaction time x group was not significant.

Table 15. Means of daily intake of mix pelleted concentrate of Sarda dairy ewes during the 3 weeks of increasing doses of furfural (F) and 2-hydroxybenzaldehyde (2-HBA) administered.

	Time[a]				Group			SEM	P-value[c]	
	24h	**48h**	**72h**	**96h**	**Control**	**F**	**2-HBA**		**time**	**group**
week1[b]	0.5	0.6	0.6	0.6	0.6[b]	0.5[a]	0.6[b]	0.09	ns	**
week 2	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.06	ns	ns
week 3	0.6[a]	0.4[b]	0.6[a]	0.6[a]	0.5	0.5	0.5	0.09	**	ns

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

[a] time: 24h = 24 hours after treatment of F or 2-HBA; 48h = 48 hours after treatment of F or 2-HBA; 72h = 72 hours after treatment of F or 2-HBA; 96h = 96 hours after treatment of F or 2-HBA;

[b] week1: low dose; week2: medium dose; week3: high dose of F or 2-HBA;

[c] the interaction time x group was not significant.

Table 16. Faecal worm egg counts of Sarda dairy ewes during furfural (F) and 2-hydroxybenzaldehyde (2-HBA) treatments.

	Dose			Treatment				P-value	
Parameter	**1**	**2**	**3**	**Control**	**F**	**2-HBA**	**SEM**	**Dose[1]**	**Treatment[2]**
epg	398.4	513.6	579.6	566.0	397.6	528.0	687.7	ns	ns

ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;

Dose 1: 72 hours after low doseof F or 2-HBA;Dose 2: 72 hours after medium dose of F or 2-HBA; Dose 3: 72 hours after high dose of F or 2-HBA;

[1]Dose*[2]Treatment= not significant.

Table 17. Regression equation, limit of detection and quantification for FA, SA, 5-HMF, F and 2-HBA in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) using a reverse phase column Varian Pursuit Xrs C18 (250 mm x 4.6 mm x 5µm).

Compounds	Rt (min)	Regression equation	Linearity range (mg/L)	R^2	LOD (S/N=3) (mg/L)	LOQ (S/N=10) (mg/L)
2- furoic acid	2.2	y= 69.72x + 91.03	0.10 - 15	0.992	0.05	0.10
salicylic acid	2.2	y=41.07x - 7.176	0.10 - 15	0.999	0.10	0.20
5-hydroxymethylfurfural	2.7	y= 294.2x – 16.82	0.10 - 15	0.999	0.05	0.13
furfural	3.8	y= 360.5x – 26.4	0.10 - 15	0.999	0.05	0.10
2-hydroxybenzaldehyde	6.3	y= 149.4x + 0.206	0.10 - 15	0.999	0.05	0.10

Table 18. Regression equation, limit of detection and quantification for5-HMF,SA, F and 2-HBA in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) using a reverse phase column Polaris C18 (300 mm x 4.6 mm x 5µm).

Compounds	Rt (min)	Regression equation	Linearity range (mg/L)	R^2	LOD (S/N=3) (mg/L)	LOQ (S/N=10) (mg/L)
5-hydroxymethylfurfural	6.1	y= 481.0x – 8.482	0.10 - 15	0.999	0.05	0.10
salicylic acid	6.8	y= 101.4x + 7.355	0.10 - 15	0.999	0.05	0.10
furfural	8.5	y= 735.6x – 165.5	0.10 - 15	0.999	0.05	0.10

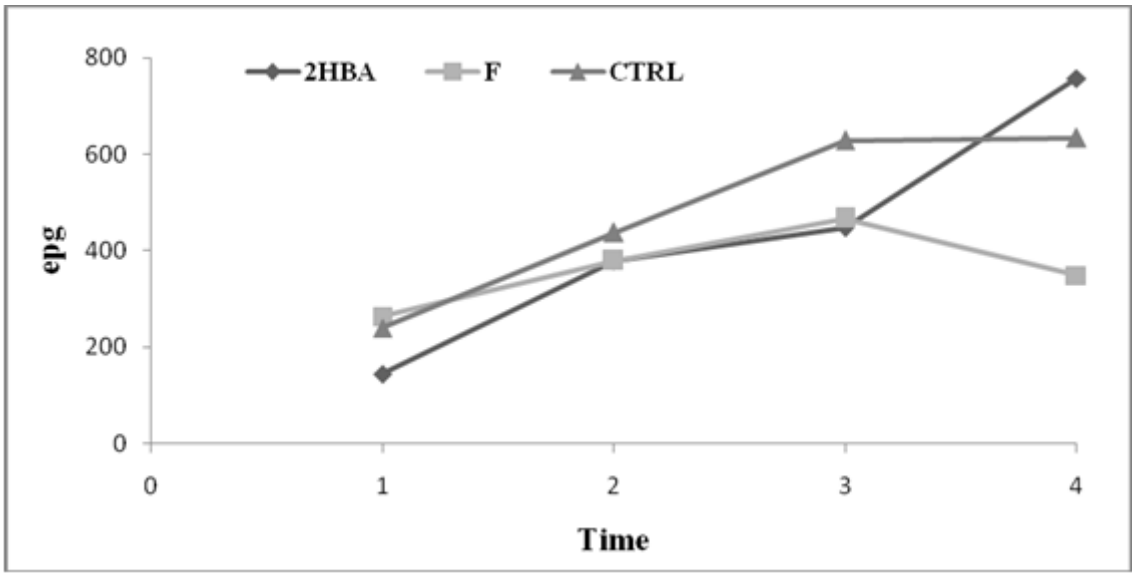
Table 19. Recovery data of F and 2-HBA in faeces (n=3).

Spiked amount (mg/mL)	% Recovery (mean ± SD)	RSD %
furfural		
15.0	80.5 ± 5.7	7.1
1.5	97.3 ± 4.5	4.6
0.1	74.2 ± 2.2	3.0
2-hydroxybenzaldehyde		
13.0	93.9 ± 8.3	8.9
1.3	91.5 ± 5.5	6.0
0.1	85.1 ± 5.1	6.0

Table 20. Recovery data of F and 2-HBA in milk (n=3).

Spiked amount (mg/mL)	% Recovery (mean ± SD)	RSD %
furfural		
15.0	88.6 ± 8.3	7.1
1.5	89.4 ± 5.5	9.9
0.1	95.4 ± 5.1	3.9
2-hydroxybenzaldehyde		
13.0	100.7 ± 5.7	8.2
1.3	110.1 ± 4.5	2.3
0.1	90.8 ± 2.2	7.0

Figure 1. Eggs per gram of the three groups (control= ctrl= furfural= F and 2 hydroxybenzaldehyde= 2-HBA) during the experimental trial.



Time 1= period before the lower dose of F and 2-HBA; Time 2= period before the medium dose of F and 2-HBA; Time 3= period before the higher dose of F and 2-HBA; Time 4= evaluation 96h after higher dose of F and 2-HBA.

Figura 2. UV spectra of 5-hydroxymethylfurfural.

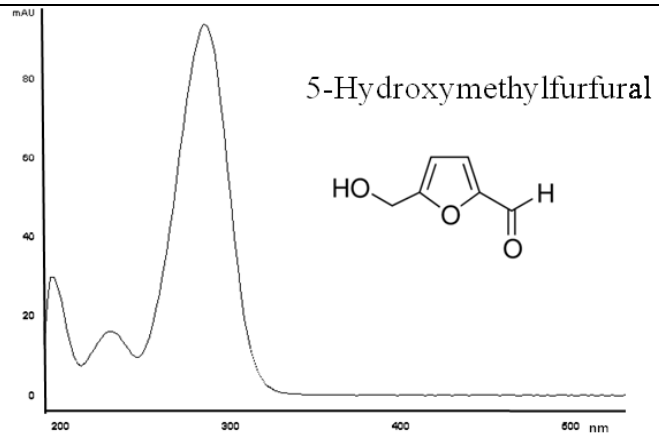


Figura 3. UV spectra of furfural.

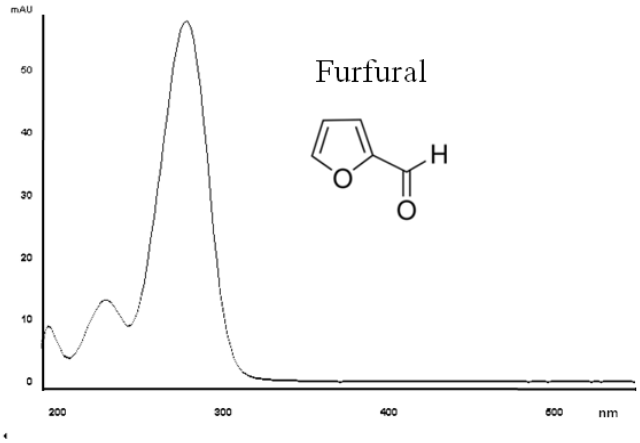


Figura 4. UV spectra of 2-furoic acid.

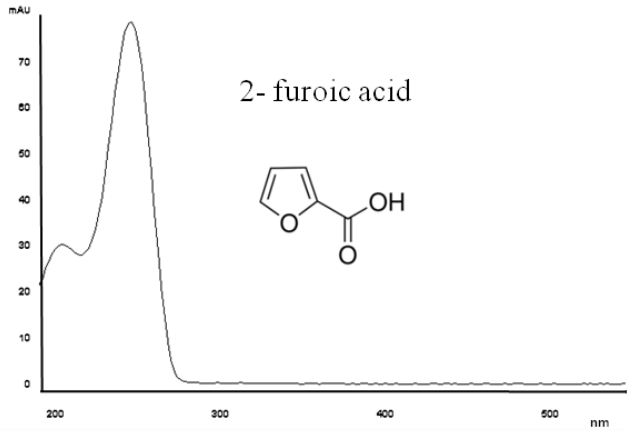


Figure 5. UV spectra of 2-hydroxybenzaldehyde.

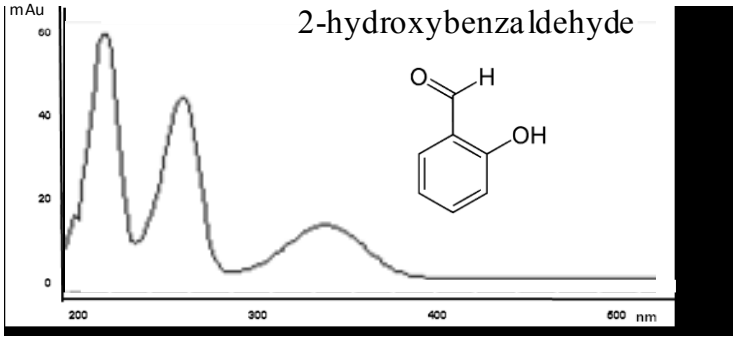


Figure 6. UV spectra of salicylic acid.

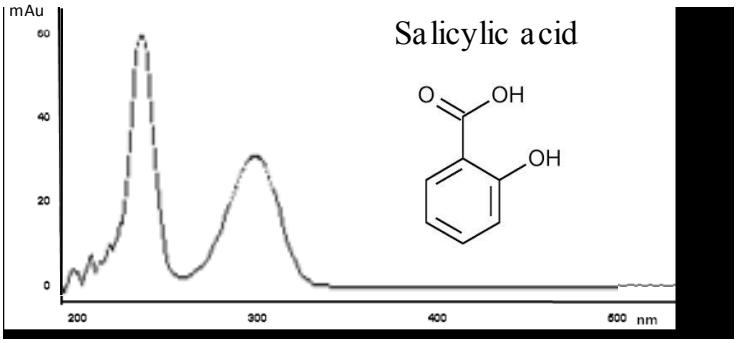
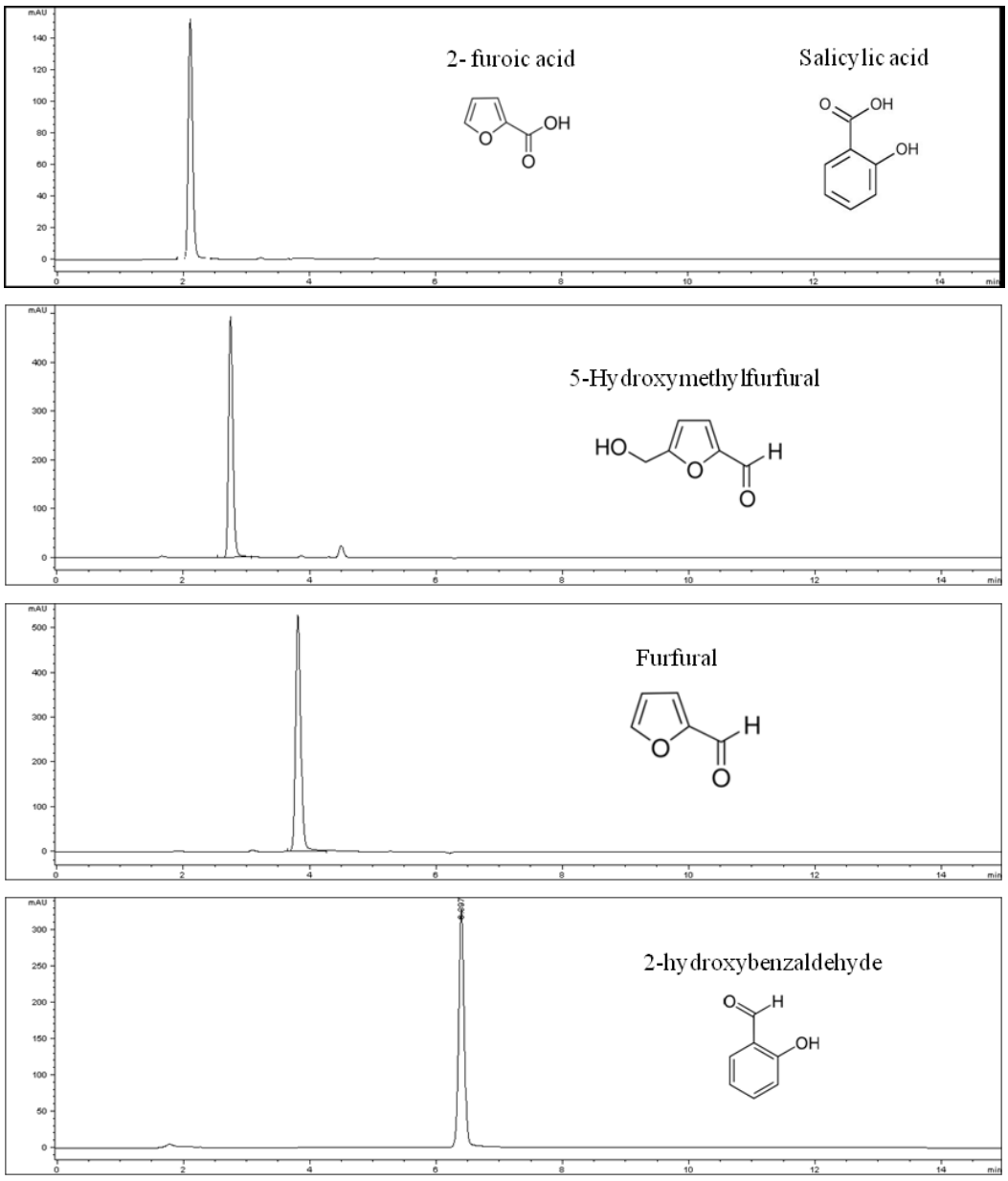


Figure 7. HPLC-DAD chromatogram for the analysis of FA, 2-HBA at 254 nm, 5-HMF, F at 280 nm and SA at 310 nm in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*).



CHAPTER 4

Parasitological effects of selected phytochemicals against gastrointestinal nematodes. Consequences on health status of Sarda dairy ewes and carry over into milk

Elisa Ortu

Parasitological effects of selected phytochemicals against gastrointestinal nematodes. Consequences on health status of Sarda dairy ewes and carry over into milk.

Abstract. In the last years, phytochemicals are considered an effective option to fight gastrointestinal nematodes infecting small ruminant herds. The aim of this work was to evaluate the anthelmintic effects of compounds of botanical origin such as, furfural, 2-hydroxybenzaldehyde and 2-undecanone against gastrointestinal nematodes in Sarda dairy ewes. Moreover, the potential carry over of these compounds into milk and the toxicological effects in Sarda dairy ewes were investigated. The experiment was carried out on 45 sheep divided into five groups: group 1 was a negative control, group 2 was a positive control and received albendazole while groups 3, 4 and 5 received a single dose of furfural, 2-hydroxybenzaldehyde and 2-undecanone, respectively. During the experiment, animals were subjected to measurements of faecal worm egg counts, blood sampling before treatment and 1, 3, 7, 14 and 21 days after drug administration. Instead, milk samples were collected 24 and 72 hours after treatment. Furfural, 2-hydroxybenzaldehyde, their metabolites and 2-undecanone were not detected in milk samples. Thus suggesting no carry over effects in the milk of these compounds. On the contrary, in milk samples belonging to albendazole group, the metabolite albendazole sulfoxide was detected 24 h after drug treatment in the range of 0.1 - 0.4 mg/kg but not in the milk collected 72 h after administration. This result was in accordance to the withdrawal period of albendazole that for sheep corresponds to 96 hours in milk for human consumption. Furthermore, all blood parameters investigated were within the normal range and no toxicological effects were observed in the ewes treated with furfural, 2-hydroxybenzaldehyde and 2-undecanone. Albendazole group showed a clear anthelmintic effect and egg per grams decreased 7 days after treatment disappearing in the following 14 days. On the other hand, furfural, 2-hydroxybenzaldehyde and 2-undecanone groups showed a trend similar to negative control: eggs per grams increased during the 7 days after drug treatment, decreased in the next week and finally increased slightly respect to the initial values. These data may be useful for further studies to optimize the anthelmintic activity of these compounds working on pharmaceutical formulation and on their mode of action.

1. Introduction

Gastrointestinal strongylosis are a serious worldwide problem that have repercussions on health status and breeding performance of small ruminants. In the last years, the interest of plant extracts to fight against gastrointestinal nematodes (GIN) is growing with the aim to contrast the development of resistance against the conventional anthelmintic drugs. Albendazole (ABZ) is a broad spectrum drug used for the control of helmintiasis in humans and ruminants. As well as other drugs used against nematodes, after several years of application, ABZ showed resistance problems in small ruminants infected by GIN. It is available in several formulations, including drenches and boluses. Marriner and Bogan (1980) demonstrated that in sheep after a single oral dose of ABZ (10 mg/kg bw), the drug was absorbed unchanged from the rumen. While, the same authors reported that albendazole sulfoxide (ABZ-SO) was detectable in plasma and in abomasal fluid 20 hours after administration. Several studies showed that ABZ is rapidly and successively metabolized to ABZ-SO and albendazole sulfone (ABZ-SO2). ABZ-SO is produced in the liver and probably in the intestinal wall (Dollery, 1999). It is the active metabolite, responsible for the therapeutic activity of ABZ (Dayan, 2003). SkrebskyCezar et al. (2010) evaluated the sensitivity of GIN infecting sheep with nine different conventional anthelmintic drugs. Especially, for ABZ-SO they detected a parasite resistance of 10% for the genera *Haemonchus* spp., *Trichostrongylus* spp. and *Ostertagia* spp. Several analytical methods are used to determine ABZ and their metabolites in different matrices. Romvfiri et al. (1998) developed a high-performance liquid chromatography (HPLC) method for the determination of ABZ-SO and ABZ-SO2 in cow milk through solid phase extraction. De Liguoro et al. (1996) studied the distribution of ABZ and its metabolites in ovine milk and products using a HPLC with a diode array detector (DAD). Moreover, González-Hernández et al. (2012) determined ABZ metabolites in plasma and cerebrospinal fluid developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. On the other hand, plants extracts appear a valid alternative to conventional anthelmintic drugs to fight GIN in small ruminants. Plants are abundant of compounds called phytochemicals that apparently not have a particular function but instead hide interesting potentiality. 2-undecanone (2-UND) is a volatile aliphatic ketone insoluble in water. It is found naturally in several food such as ginger (Balakrishnan, 2005), bananas, cloves and

strawberries. Due to its strong odour, 2-UND is used also as animal repellent for the protection of home garden and hornamentals (EFSA, 2012). In addition, it is employed in the perfumery industry as fragrance additive to soaps, detergents, and perfumes (The Human Metabolome Database). 2-UND is the main volatile component of *Ruta chalepensis* L, (Ntalli et al., 2011; Tounsi et al., 2011; Haddouchi et al. 2013) *Ruta Montana* L. and *Ruta graveolens* L. essential oils (Soleimani et al., 2009; Ferhat et al., 2014). Moreover, it is one of the main component of *Houttuyniacordata thunb* (Liang et al., 2005). Furthermore, 2-UND was detected in dairy products as volatile flavour components (Iličić et al., 2012), especially Vazquez-Landaverde et al. (2005, 2007) identified it through the use of solid-phase microextraction fiber. In the previous chapters of this PhD thesis, were studied the *in vitro* activity of selected phytochemicals against GIN of sheep. Furfural (F), 2-hydroxybenzaldehyde (2-HBA) and 2-UND showed an interesting anthelmintic activity with an $EC_{50/24h}$ of 1.83, 2.19 and 0.88 mg/mL, respectively. Later, F and 2-HBA were administered in dairy ewes using increasing doses. Overall, the results indicated no significant effects on blood and no carry over into milk. Thanking into account these previous studies, aim of this work was evaluate the anthelmintic activity of F, 2-HBA using higher doses than in the previous experimental trial. Moreover, the anthelmintic activity of 2- UND against GIN in dairy ewes was investigated. In addition, objective of this research was to study the possible carry over into milk of the drugs administered and of their metabolites and monitor the possible toxicological effects of these compounds.

2. Material and methods

Chemicals

Standards of 5-hydroxymethylfurfural (5-HMF), furfural (F), 2-furoic acid (FA), 2-HBA, salicylic acid (SA), 2-UND and ABZ-SO of purity greater than 98%, as well as acetonitrile, dichlormethan, ethyl acetate, hexane, methanol, oxalic acid, sodium hydroxide, trichloroacetic acid (TCA) and ammonium formate were purchased from Sigma-Aldrich (Milano, Italy). Water used was purified on a Milli-Q apparatus. Acetonitrile, dichlormethan, ethyl acetate, hexane and methanol used were HPLC grade while formic acid was LC-MS grade.

Experimental design

The experiment was carried out in 45 Sarda multiparous dairy ewes (aged of 4 years, weighing between 28.4 and 41.0 kg) at AGRIS farm located in Bonassai, Sassari, Italy. The trial was carried out between June and July 2013 following the European Commission Council Directive that regulates the use of animals for experimental and other scientific purposes. During the experiment, each ewe received a controlled diet consisting on fresh chopped alfalfa (3 kg), mix pelleted concentrate (0.6 kg) and dry rye grass *ad libitum*. Furthermore, the ewes had water *ad libitum* and were maintained indoors during the first five days after drug treatment with the aim to reduce further GI infestations. After seven days of acclimation, animals were divided into five groups:

- group 1 (control): was a negative control (i.e. not treated);
- group 2 (albendazole): was a positive control. Ewes received a sinlge oral dose of the conventional anthelmintic drug albendazole (Valbazen ®, Pzifer, New York, United States);
- group 3 (furfural): ewes received F in a single-dose;
- group 4 (2-hydroxybenzaldehyde): ewes received 2-HBA in a single-dose
- group 5 (2-undecanone): ewes received 2-UND in a single-dose.

During the experiment, animals were subjected to measurements of faecal worm egg

counts (FWECs), body weight (BW), body condition score (BCS) and milk and blood samples were collected. Moreover, each group were statistically balanced according to FWECs, BCS and BW.

*Drug formulation***.**

The F, 2-HBA and 2-UND doses were dissolved in 10 mL of sunflower oil and orally administered. Animals of group 1 received only 10 mL of sunflower oil, animals of group 2 received 4 mL/20 kg bw of Valbazen ® (corresponding to 3.75 mg/kg bw of albendazole), while the groups 3, 4, 5 received a single oral dose of F, 2-HBA and 2-UND: 20, 110 and 125 mg/kg bw respectively. Before drug administration, samples were vigorously shaken with a vortex apparatus to favourite the total dissolution of the active ingredient. After dosing, the test tubes containing the drug were re-rinsed with 10 mL of distilled water and administered to the animals.

Blood samples.

Individual blood samples were collected by jugular vein before the administration and 1, 3, 7, 14 and 21 days after drug administration. Test tubes with the anticoagulant ethylenediaminetetraacetic acid (EDTA) were used for the complete blood count (CBC) analysis, while for the biochemical profile test tubes without anticoagulant were used. Samples were stored at 4°C and then were delivered to laboratory of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Italy) for immediate analysis.

Milk samples.

Ewes were milked before treatment and 24 and 72 hours after the drug administration. Samples were collected with the aim of evaluating and quantifying the possible residual drugs administered and were stored at -80°C until use. Within five days of drug administration, the milk was discarded to avoid drug residues in milk for human consumption.

Parasitological samples.

Faecal samples were collected from the rectum of Sarda dairy ewes an hour before treatment and 7, 14, 21 days after drug administration. FWECs were performed according to a modified Mc Master technique (Raynaud, 1970; Rossanigo and Gruner, 1991). Moreover, the third stage larvae (L_3) of Strongyle type nematodes were obtained by coproculture (Sargison, 2008) and larvae were identified by the aid of an optic microscope as the following GIN species in function of the groups: *Trichostrongylus* spp. (73-78%), *Teladorsagia* spp. (5-10%), *Haemonchus contortus* (5-14%) and *Chabertia ovina* (3-18%).

Clinical observations.

During the experiment ewes were carefully monitored by a veterinarian in order to detect any adverse reaction to the treatment.

Chemical Analysis

Blood analysis.

Hemogram parameters such as red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume of red blood cells (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH); total platelets (PLT), and total white blood cells count values such as white blood cells (WBCB), neutrophils cells (NEUTS), lymphocytes cells (LYMPHS), monocytes cells (MONOS), eosinophils cells (EOS) and basophils cells (BASOS) were analyzed by using a Laser CyteAnalyzer (IDEXX Laboratories, Milan, Italy). On the other hand, the biochemical parameters, such as albumin (ALB), alkaline phosphatase (ALP), total bilirubine (BT), creatinine (CRE), gamma glutamiltranspeptidase (GGT), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), urea (UR) and total protein (PROT), were determined by a clinical analyzer spectrophotometric method (Dimension RXL Chemistry Analyzer, Dade Behring, Munich, Germany).

Milk HPLC analysis.

The analysis for the F and 2-HBA groups were performed on HPLC using an Agilent Technologies 1100 series (Waldbronn, Germany) equipped with a quaternary pump, autosampler, a degasser system and a diode array detector (DAD). A reverse phase column Varian Pursuit Xrs C18 (250 mm x 4.6 mm x 5µm) was employed and a guard column of the same material was used. The gradient program was as follows: starting from aqueous ammonium formate and acetonitrile (50:50 *v/v*) to aqueous ammonium formate and acetonitrile (35:65 *v/v*) in 8 min and then decreasing to 50% of aqueous ammonium formate in 7 min. The flow rate was 1.0 mL/min and the injection volume was 50 µL. The UV- DAD analysis was performed at a wavelength of 280 nm for F, 5-HMF, 254 nm for FA, 2-HBA and 310 nm for SA. Standard stock solutions were prepared daily in a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*) and stored at -20°C until use. Samples for F, 5-HMF and FA determination were prepared dissolving 5g of milk into 1.5 mL of 0.15 M oxalic acid (freshly prepared) and 1 mL of 40 % (*w/v*) TCA in a sealed tube to prevent evaporation. Each sample was mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 15 min. at 3000 rpm (ALC 4218 centrifuge. Milan, Italy). The two phases were separated, 3 mL of 4 % (*w/v*) TCA were added to solid residue obtained and the sample was mixed for 10 minutes and centrifuged for 15 min. at 3000 rpm. TCA extract was transferred in a 25 mL volumetric flask and was filled the volume with 4 % (*w/v*) TCA. Later, samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. The sample preparation was in accordance to Albalà-Hurtado et al. (1997) with slight modifications. On the other hand, samples for 2-HBA and SA determination were prepared dissolving 2g of milk into 2 mL of ethyl acetate and a sealed tube to prevent evaporation. Each sample was mixed with a rotary shaker for 15 minutes (Falc Instrument. Bergamo, Italy) and then centrifuged for 30 min at 4000 rpm (ALC 4218 centrifuge. Milan, Italy). After, 100 µL of the upper phase were dissolved into 900 µL of a mixture consisting of acetonitrile and aqueous ammonium formate 10 mM (50:50, *v/v*). Samples were filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for HPLC analysis. An aliquot of 100 µL of F and 2-HBA at desired concentration was added to 5g of milk and extracted using the method previously indicated for milk samples. Three

replicates of each matrix were analyzed. The fortification levels used were in the range of 0.1 - 15 mg/L for F and 0.1 - 13 mg/L for 2-HBA. The recoveries were calculated by interpolation in calibration curves of matrix matched standards. Moreover, 5-HMF, F, FA, 2-HBA and SA were quantified in milk by interpolation in calibration curves of matrix matched standards in the range of 0.1-15 mg/L. Furthermore, to evaluate the sensitivity of the method, the detection limit (LOD) and quantification limit (LOQ) were estimate.

Milk GC-MS analysis

Milk samples from sheep treated with 2-UND were diluted 1:10 with hexane, filtered through a 0.45 µm filter (Agilent, 25 mm, nylon membrane) and injected for GC-MS analysis using a method according to Ntalli et al. (2011) and Caboni et al. (2012) with slight modifications. The chromatographic separation and identification of the main components were performed on a Trace GC ultra gas chromatograph (Thermo Finnigan, San Jose, CA) coupled with a Trace DSQ mass spectrometry detector, a split-splitless injector an Xcalibur MS platform. The column used was polar fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30 m x 0.25 mm; film thickness, J&W 0.25 µm Scientific Fisons, Folsom (CA). The injector and transfer line were at 250 °C, while the oven operating conditions were as follows: from 50°C (held for 5 min) to 280°C at 9 °C/min heating rate and kept at this temperature 8 for min. The carrier gas was helium with a constant flow rate of 1mL/min; the sample (1 µL) was injected in splitless mode (60 s). Mass spectrometry acquisition was carried out using the following conditions: ionization voltage, 70 eV; scan rate 1.6 scan/s; mass range, 50-550 amu. Standard stock solutions of 2-UND were prepared daily in hexane and injected for calibration. An aliquot of 100 µL of 2-UND at desired concentration was added to 900 µL of milk and extracted with hexane using the method previously indicated. Three replicates of each matrix were analysed. The fortification levels used were in the range of 0.05- 100 mg/L. The recoveries were calculated by interpolation in calibration curves of matrix matched standards. 2-UND was quantified in milk by interpolation in calibration curves of matrix matched standards in the range of 0.05-100 mg/L. Moreover, to evaluate sensitivity of the method, the detection limit (LOD) and quantification limit (LOQ) were estimate.

Milk LC-MS analysis.

Milk samples belonging to ABZ group were analysed using Varian tandem mass spectrometer (Palo Alto, CA; USA) equipped with a ProStar autosampler, two ProStar pumps and a 1200L triple quadrupole. An electrospray ionization source was used operating in the positive-ion detection mode. Varian MS workstation version 6.7 software was used for data acquisition and processing. The chromatographic separation was performed on a Varian column Pursuit XRs C18 (150 x 2.0 mm, 5 µm). The mobile phasesused were (A) methanol and (B) formic acid 20 mM in accordance with González-Hernández et al. (2012). The solvent gradient (*v/v*) was started from 30 % B and reaching 100 % B in 10 min, maintaining 100% B for 5 min for a total run of 20 min. The mobile phase was pumped at a flow rate of 0.3 mL/min and the injection volume was10 µL. The electrospray capillary potential was set to 51 V and the shield at 600 V. Nitrogen gas was used for nebulising the HPLC eluent and for drying the solvent. The temperature of the ESI housing was kept at 65°C while the drying gas at 200°C. Milk samples were prepared in according to Abdel-tawab et al. (2009) and DeRuyck et al. (2002) with slight modifications. 100 µL of sodium hydroxide 0.4 N was added to 1 mL of milk previously sonicated for 10 minutes in a test tube and mixed with a vortex apparatus for 1 minute. Then 6 mL of ethyl acetate was added and mixed for 1 minute and the mixture was centrifuged for 10 min. at 3000 rpm (ALC 4218 centrifuge. Milan, Italy). The upper phase was recovered and the solid residue was extracted a second time using sodium hydroxide and ethyl acetate. Then, the two upper phases were mixed and dried under nitrogen, reconstituted with 1 mL of methanol-milliQ water (1% formic acid) 70:30 (*v/v*). The sample was filtered through a filter of 0.45 µm (Agilent, 25 mm, nylon membrane) and injected for LC-MS analysis. ABZ-SO detection was performed using a multiple reaction monitoring (MRM) with the following transitions: m/z 282.2 →239.6, m/z 282.2 →207.8 and m/z 282.2 →158.8 with a scan time of 0.4 sec per transition. The collision energies used were 36, 24 and 16 V, respectively. Adequate amounts of ABZ-SO were dissolved in 1 mL mixture consisting of methanol and formic acid 20 mM (70:30, *v/v*). An aliquot of 100 µL of ABZ-SO at desired concentration was added to 900 µL of milk and extracted using the method previously indicated for milk samples. Three replicates of each matrix were analyzed. The fortification levels used were in the range of 0.01 - 30 mg/L. The

recovery were calculated by interpolation in calibration curves of matrix matched standards. Stock solutions were diluted with the same mixture and injected in triplicate for the preparation of calibration curves. ABZ-SO in milk was quantified by interpolation in calibration curves of matrix matched standards in the range of 0.01-30 mg/L. Samples were fortified at the desired level of ABZ-SO and directly injected for LC-MS analysis after dilution with blank sample. Calibration curves were created by plotting the concentration of these compounds against the standard peak area following the standard external method. The linearity of the method was demonstrated using blank sample spiked with standard of ABZ-SO at concentration level of 0.01-30 mg/L. Moreover, to evaluate the sensitivity of the method, the detection limit (LOD) and quantification limit (LOQ) were estimate.

Statistical analysis

Blood data

Blood serum biochemistry, hemogram, total and differential white blood cells count data were analyzed by a general linear model ANOVA design. The treatment and time effects were analyzed. For all blood parameters analysed, the five times used corresponded to the samples collected 1, 3, 7, 14 and 21 days after drug treatment. Concerning to the statistical blood serum biochemistry analysis, were detected significant differences between the data relating to the period before drug treatment. For this reason, it was essential covariate the data corresponding to 1, 3, 7, 14 and 21 days after treatment with the data prior to treatment (Table 1). On the contrary, for hemogram and, the total and differential white blood cells count analysis, the data before drug treatment were not considered because no significant differences were observed (Tables 2 and 3).

Parasitological data

Parasitological data were analysed by a general linear model ANOVA design. The treatment and time effects were analysed. The three times indicated, corresponded to the faecal samples collected at 7, 14 and 21 days after drug treatment. Furthermore, the data before treatment were not considered because no significant differences were observed. The statistical analysis is shown in Table 4.

3. Results and discussion

Drug formulation

F, 2-HBA and 2-UND formulations were prepared taking into account their water solubility and the LD_{50}. F and 2-HBA have a partition-coefficient (logP) of 0.41 and 1.81, respectively. Therefore, F is more soluble in water than 2-HBA with a solubility of 83 g/L at 20°C; while 2-HBA have a solubility equal to 14 g/L at 20°C. On the other hand, 2-UND is insoluble in water but soluble in organic solvent such as ethanol, ether, acetone and chloroform (Lide and Milne, 1994) while water solubility is 20 mg/L at 25°C (EPA; 2004) and logP equal to 4.09. Considering these information, it was decided to prepare all drug formulations using sunflower oil to increase the solubility of these compounds. Moreover, the oral LD_{50} in rats for F, 2-HBA and 2-UND were 100-127 mg/kg bw (Sigma Aldrich, Milan Italy; Jenner *et al.,* 1964), 520 mg/kg bw rats (Merck, Italy) and 5g/kg bw (O'Neil, 2001), respectively. The evaluation of the LD_{50} for 2-UND was performed using rat data because there are no information in literature about oral acute toxicity in small ruminants. On the other hand, in the previous study in Sarda dairy ewes, F and 2-HBA were used at dosage of 1/80, 1/40 and 1/20 respect to oral LD_{50} in rats and no were observed toxicological effects. For this reason, it was decided to use doses of F and 2-HBA higher than the previous study. Therefore, the single dose of F, 2-HBA used was 1/5 compared to the oral LD_{50} in rats, whereas for 2-UND was used 1/40 respect to oral LD_{50}. For the calculation of the single dose of drug administered in the ewes, for each group their mean of body weight was considered.

Chemical composition

Milk analysis

As well as faecal and milk samples analysed in the previous chapter, the milk analysis were carried out using a Varian Pursuit Xrs C18 column. Consequently, the UV wavelengths, the HPLC retention times, the LOD and LOQ were the same (Table 5). FA, SA, 5-HMF, F and 2-HBA were not detected in milk samples collected 24 and 72 hours after drug treatment. In addition, traces of 2-UND were searched in milk samples by GC/MS. 2-UND showed a retention time of 16.0 minutes, LOD and LOQ were 0.05 and 0.25 mg/L, respectively (Table 6 and Figure 1). 2-UND was not detected in milk samples collected 24 and 72 hours after treatment. These facts for F, 2-HBA and 2-UND groups led us to ruled out a carry-over phenomenon in milk. On the other hand, in the ewes treated with 3.75 mg/kg bw of ABZ, the metabolite ABZ-SO was detected by LC-MS at 11.6 minutes with a LOD and LOQ of 0.005 and 0.01 mg/L, respectively (Table 7). The chromatogram and the ion transitions were shown in the Figure 2. ABZ-SO were detected and quantified in the samples collected 24h after drug treatment in the range of 0.1-0.4 mg/L but not in the milk collected 72 h after administration. Considering milk density, these results were in accordance to De Liguoro et al. (1996) that administered to Laticauda sheep 12.5 mg/kg bw of ABZ obtaining average levels of ABZ-SO in milk equal to 1.8 mg/kg 24 h after treatment and 0.06 mg/kg 48 h after drug administration. These levels are in accordance with De Liguoro. Furthermore, the absence of ABZ-SO in milk collected 72 h after treatment was in accordance to the sheep withdrawal period of 96 hours (Ministero della Salute, 7 Giugno 2012).

Statistical analysis

Blood data

Blood serum biochemistry data were analyzed evaluating five different times corresponding to the samples collected 1, 3, 7, 14 and 21 days after drug treatment (Table 1). The statistical differences through groups were analyzed during the five times of sampling. For ALB, BT, CRE, GGT and GOT were not observed significant differences between times and treatments. On the other hand, there were detected

differences (P≤0.01) for ALP, GPT and PROT between times and treatments. For ALP, the parameters at 1, 3 and 7 days after treatment were similar (in the range of 76.4-83.3 U/L) and increased in the samples corresponding to 14 and 21 days after drug administration, reaching the range of 111.5-121.1 U/L. Moreover, for all groups the ALP values were similar, except for ABZ treated animals in which the parameter was lower if compared with the other groups. For GPT values, the first three times were similar (range 36.1-37.2 U/L) and increased in the last two sampling times (40.1-41.3 U/L). 2-UND showed the lower GPT value (36.3 U/L) while highest was the control (39.9 U/L). The other treatments were inside this range, especially 2-HBA and ABZ were similar to control while F was similar to 2-UND. On the other hand, for PROT the values were the same in the samples collected 1 and 7 days after treatment (7.6 g/dL) while decreased in the last three times (range 7.0-7.2 g/dL). F showed the lower value (7.0 g/dL) while ABZ the highest (7.5 g/dL). 2-UND (7.1 g/dL) group exhibited a value near to F and 2-HBA (7.3 g/dL) while the control (7.4 g/dL) was similar to ABZ. Urea levels showed significant differences between times (P≤0.01) with the highest value (56.1 mg/dL) 1 day after treatment and the lower value 14 days after drug administration (39.3 mg/dL). The groups showed significant differences (P≤0.05), especially control, F and ABZ revealed similar values (48.5-48.6 mg/dL), while 2-HBA exhibited the lower value (47.2 mg/dL) and 2-UND the highest (51.5 mg/dL). Compared to the previous experiment, significant differences were no detected in ALB and GOT parameters between control, F and 2-HBA. In addition, hemogram (Table 2) and, total and differential white blood cells count (Table 3) were statistically analysed considering five times and five groups. In this case was not essential covariate the data because the data before treatment no showed significant differences. Concerning to hemogram, RBC, HGB, HCT and MCHC values showed statistical differences for time and treatment parameters (P≤0.01). On the contrary, MCV showed differences only for the time parameters (P≤0.01) while PLT revealed statistical differences both for time (P≤0.01) and for treatment (p≤0.05). In particular, for PLT parameter, 2-UND showed the highest value (799.1 x 103 cells/µL) while ABZ the lower (708.6 x 103 cells/µL). For the total and differential white blood cells count, BASOS and WBCB showed significant differences in time and treatment (P≤0.01), LYMPHS and MONOS only for time (P≤0.01) while EOS only for treatment (P≤0.05). Moreover, NEUTS exhibited significant differences both for the time (P≤0.01) and for the treatment (P≤0.05) values.

In all cases, the parameters analysed were within the normal range.

Parasitological data

FWECs data were statistically analysed by a general linear model ANOVA design (Table 4). Time and treatment values were studied; both exhibited significant differences with a $P \leq 0.05$ and $P \leq 0.01$ respectively. Respect to time, the eggs per gram of feces (epg) mean was high (2302 epg) 7 days after drug treatment, decreased 14 days after treatment (1041 epg) and increased slightly 21 days after administration (1396 epg). With regard to treatments, F showed the highest value (2431 epg) while ABZ exhibited clearly an anthelmintic effect with a epg mean equal to 77.1. The control and 2-UND groups revealed a mean values similar to F (1891 and 2008 epg, respectively) while 2-HBA showed a mean epg value between the other groups. The figure 3 showed the trend of epg mean in the five groups during the three weeks of experiment. The groups at the beginning of the treatment had indicatively similar epg mean values, except ABZ that had values slightly higher than the other groups. For ABZ, as revealed the statistical analysis, was clear an anthelmintic effect. The conventional drug, 7 days after treatment showed a fast reduction of epg mean disappearing and remaining stable during the following 14 days. On the other hand, in the other groups, 7 days after drug treatment, epg mean increased, especially in F group and decreased the next week with values similar to the beginning of the experiment. Finally, during the third week after drug treatment, the epg mean values increased slightly.

Clinical observations

Ewes during the experiment were kept under control to monitor possible negative and toxicological effects caused by administered drugs. Health status of the animals were monitored with the help of a veterinarian, before drug treatment and weekly during the 21 days after treatment. No animal showed toxicological symptoms.

4. Conclusions

F, 2-HBA, their metabolites and 2-UND were no detected in milk samples. Thus suggesting no carry over effects in the milk. On the contrary, ABZ-SO was detected in milk samples 24 hours after treatment but was within the official withdrawal period applied in milk for human consumption. Considering all blood parameters, the administration of F, 2-HBA and 2-UND showed no toxicological effects. Moreover, no particular diseases were observed in animals treated with these compounds. ABZ showed a clear anthelmintic effect while F, 2-HBA and 2-UND revealed a trend similar to control group. In the future, we will investigated the metabolism of these compounds. For this reason, is essential to find an optimal pharmaceutical formulation that protect the drug by possible rumen degradation but at the same time effective against GIN located in the abomasum, large and small intestine. Furthermore, to reach these objectives, is essential to study the pharmaceutical formulation in function on the chemical structure of the drug and the drug mode of action. In conclusion, the anthelmintic effects showed by F, 2-HBA and 2-UND in this study could be improved working on dosage form and on their mode of action.

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6. References

Abdel-tawab A. M, Bradley M., Ghazaly E. A., Horton J., El-Setouhy M. 2009. Albendazole and its metabolites in the breast milk of lactating women following a single oral dose of albendazole. *Br. J. Clin. Pharmacol*. 68, 737–742.

Albalá-Hurtado S., Veciana-Nogués M. T., Izquierdo-Pulido M., Vidal-Carou M. C. 1997. Determination of Free and Total Furfural Compounds in Infant Milk Formulas by High-Performance Liquid Chromatography. *J. Agric. Food Chem.*, 45, 2128–2133.

Balakrishnan K. V. 2005. Ginger: The Genus Zingiber. Edited by Ravindran P. N., Nirmal Babu K. CRC Press, Boca Raton, Florida. 11, 408-410.

Caboni P., G. Ntalli N., Aissani N., Cavoski I., Angioni A. 2012. Nematicidal activity of (E,E)-2,4-Decadienal and (E)-2-Decenal from *Ailanthus altissima* against *Meloidogyne javanica*. *J. Agr. Food Chem.*, 60, 1146−1151.

Dayan A.D. 2003. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. *Acta Trop.* 86, 141–159.

De Liguoro M., Longo F., Brambilla G., Cinquina A., Bocca A., Lucisano A. 1996. Distribution of the anthelmintic drug albendazole and its major metabolites in ovine milk and milk products after a single oral dose. *J Dairy Res.*, 63, 533-42.

De Ruyck H., Daeseleire E.; De Ridder H. 2002. Development and validation of a liquid chromatographic-electrospray tandem mass spectrometric multi-residue method for anthelmintics in milk. *J. Chromatogr. A*, 976, 181-194.

Dollery C.T. 1999. Albendazole. In: Therapeutic Drugs, Second ed. Churchill Livingstone, Edinburgh, Volume 1, 51-53.

EFSA Journal 2012; 10, 2495.

Ferhat M., Kabouche A., Kabouche Z. 2014. Comparative compositions of essential oils of three Ruta species growing in different soil. *J. Mater. Environ. Sci.*, 5, 735-738.

González-Hernández I., Ruiz-Olmedo M. I, Cárdenas G., Jung-Cook H. 2012. A simple LC-MS/MS method to determine plasma and cerebrospinal fluid levels of albendazole metabolites (albendazole sulfoxide and albendazole sulfone) in patients with neurocysticercosis. *Biomed. Chromatogr*., 26, 267–272.

Haddouchi F., Chaouche T. M., Zaouali Y., Ksouri R., Attou A., Benmansour A. 2013. Chemical composition and antimicrobial activity of the essential oils from four Ruta species growing in Algeria. *Food Chem.*, 141, 253-258.

Iličić M. D., Milanović S. D., Carić M. D., Kanurić K. G., V. R. Vukić, D. V. Hrnjez Ranogajec M.I. 2012. Volatile compounds of functional dairy products. *Acta Periodica Technologica*, 43, 11-19.

Jenner P. M., Hagan E. C., Taylor J. M., Cook E. L., Fitzhugh O. G. 1964. Food flavourings and compounds of related structure. I. Acute oral toxicity. *Food and Cosmet. Toxicol.*, 2, 327–343.

Liang M., Qi M., Zhang C., Zhou S., Fu R., Huang J. 2005. Gas chromatography–mass spectrometry analysis of volatile compounds from *Houttuyniacordata thunb* after extraction by solid-phase microextraction, flash evaporation and steam distillation". *Anal. Chim. Acta*, 531, 97–104.

Lide D. R., Milne G. W. A. 1994. Handbook of Data on Organic Compounds. Volume I. 3rd ed. CRC Press, Inc. Boca Raton , FL. V5, 5176.

Marriner S. E., Bogan J. A. 1980. Pharmacokinetics of albendazole in sheep. *Am. J. Vet. Res.*, 41, 1126-1129.

Ministero della Salute, GU n.131 del 7-6-2012. Modificazione dell'autorizzazione all'immissione in commercio del medicinale per uso veterinario «Valbazen».

Ntalli N. G., Manconi F., Leonti M., Maxia A., Caboni P. 2011. Aliphatic Ketones from *Ruta chalepensis* (*Rutaceae*) Induce Paralysison Root Knot Nematodes. *J. Agr. Food Chem.*, 59, 7098–7103.

O'Neil M. J. 2001. An Encyclopedia of Chemicals, Drugs, and Biologicals. The Merck Index - 13th Edition. Whitehouse Station, NJ: Merck and Co., Inc., p. 1088.

Raynaud J. P. 1970. Etude de l'efficacitè d'une technique de coproscopie quantitative pour le diagnostic de routine et le controle des infestations parasitaires des bovins, ovins, èquins et porcins. *Ann. Parasitol.*, 45, 321-342.

Romvfiri Z. , Fekete J., Kemény S., Pokol G., Gebefiigi I., Kettrup A. 1998. Determination of two Metabolites of Albendazole, Albendazole-Sulfoxide and Albendazole-Sulfone in Cow's Milk Using an HPLC Method- A Systematic Approach to Optimise Extraction Conditions. *Chromatographia*, 48, 11-12.

Rossanigo C. E., Gruner L. (1991). Accuracy of two methods for counting eggs of sheep nematode parasites. *Vet. Parasitol.*, 39,115-121.

Sargison N. 2008. Sheep Flock Health: A Planned Approach. 1st ed. Blackwell, Oxford, UK.

Skrebsky Cezar A., Toscan G., Camillo G., Sangioni L. A., Olmedo Ribas H., Silveira Flôres Vogel F. 2010. Multiple resistance of gastrointestinal nematodes to nine different drugs in a sheep flock in southern Brazil. *Vet. Parasitol.*, 173, 157–160.

Soleimani M., Azar P. A., Saber-Tehrani M., Rustaiyan A. 2009. Volatile Composition of *Ruta graveolens* L. of North of Iran. *World Applied Sciences Journal*, 7, 124-126.

Tounsi, M. S., Wannes, W. A., Ouerghemmi, I., Msaada, K., Smaoui, A., Marzouk, B. 2011. Variation in essential oil and fatty acid composition in different organs of cultivated and growing wild *Ruta chalepensi s*L. *Ind. Crop. Prod.*, 33, 617-623.

Vazquez-Landaverde P. A., Qian M. C., Torres J. A. 2007. Kinetic Analysis of Volatile Formationin Milk Subjected to Pressure-AssistedThermal Treatments. *J. Food Sci.*, 72, 389-398.

Vazquez-Landaverde P. A., Velazquez G., Torres J. A., Qian M. C. 2005. Quantitative Determination of Thermally Derived Off-Flavor Compoundsin Milk Using Solid-Phase Microextraction and Gas Chromatography. *J. Dairy Sci.*, 88, 3764–3772.

http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm

http://www.hmdb.ca/metabolites/HMDB33713

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Table 1. Blood serum biochemistry of Sarda dairy ewes during the experiment.

	Time					Treatment						P-value	
Parameter	**1**	**2**	**3**	**4**	**5**	**Control**	**F**	**2-HBA**	**2-UND**	**ABZ**	**SEM**	**Time**	**Treat.[a]**
ALB, g/dL	2.8	2.8	2.7	2.7	2.7	2.8	2.7	2.78	2.6	2.8	0.18	ns	ns
ALP, U/L	83.3^{y}	82.6^{y}	76.4^{y}	111.5^{x}	121.1^{x}	97.6^{a}	99.9^{a}	105.0^{a}	97.9^{a}	74.7^{b}	16.9	**	**
BT, mg/dL	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.38	0.3	0.35	0.08	ns	ns
CRE, mg/dL	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.10	ns	ns
GGT, U/L	99.7	96.5	89.5	95.6	97.0	94.6	95.7	94.7	102.3	98.1	11.2	ns	ns
GOT, U/L	174.6	178.8	162.9	177.9	175.3	171.4	169.0	192.2	173.5	163.4	46.6	ns	ns
GPT, U/L	36.1^{y}	37.2^{y}	36.6^{y}	41.3^{x}	40.1^{x}	39.9^{a}	37.0^{bc}	39.0^{ab}	36.3^{c}	39.1^{ab}	3.78	**	**
PROT, g/dL	7. 6^{x}	7. 6^{x}	7.0^{y}	7.0^{y}	7.2^{y}	7.4^{a}	7.0^{c}	7.3^{ab}	7.1^{bc}	7.5^{a}	0.40	**	**
UREA, mg/dL	56.0^{x}	49.2^{y}	55.6^{x}	39.3^{w}	44.0^{z}	48.5^{ab}	48.5^{ab}	47.2^{b}	51.5^{a}	48.6^{ab}	6.72	**	*

[a.] Treat.= Treatment;
Different letters mean significant differences;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
F= furfural; 2-HBA= 2-hydroxybenzaldehyde; 2-UND: 2-undecanone; ABZ: albendazole;
Time1: 1 days after F, 2-HBA, 2-UND and ABZ treatment; Time2: 3 days after F, 2-HBA, 2-UND and ABZ treatment; Time3: 7 days after F, 2-HBA, 2-UND and ABZ treatment, Time4: 14 days after F, 2-HBA, 2-UND and ABZ treatment; Time5: 21 days after F, 2-HBA, 2-UND and ABZ treatment;
ALB = Albumin, ALP = Alkaline Phosphatase, BT = Total Bilirubine, CRE = Creatinine, GGT = Gamma glutamiltranspeptidase, GOT = Glutamic Oxaloacetic Transaminase, GPT= Glutamic Pyruvic Transaminase, PROT = Total Protein.

Table 2. Hemogram of Sarda dairy ewes during the experiment.

	Time					Treatment						P-value	
Parameter	**1**	**2**	**3**	**4**	**5**	**Control**	**F**	**2-HBA**	**2-UND**	**ABZ**	**SEM**	**Time**	**Treat.[a]**
RBC, x 10^6 cells/µL	8.2^{xy}	8.7^{x}	7.5^{y}	7.4^{y}	8.2^{xy}	8.4^{a}	7.1^{b}	7.4^{b}	8.5^{a}	8.6^{a}	0.87	**	**
HGB, g/dL	9.7^{x}	10.0^{x}	8.5^{y}	8.2^{y}	8.5^{y}	9.1^{a}	8.4^{b}	9.3^{a}	9.0^{a}	9.2^{a}	0.82	**	**
HCT, %	28.4^{x}	28.9^{x}	24.9^{y}	24.6^{y}	25.0^{y}	26.8^{a}	24.9^{b}	26.9^{a}	26.2^{ab}	27.1^{a}	2.56	**	**
MCV, fl	29.6^{xy}	29.2^{y}	29.7^{xy}	30.6^{x}	30.8^{x}	30.0	30.1	29.5	29.7	30.5	0.67	**	ns
MCH, pg	10.1	10.1	10.2	10.2	10.4	10.1	10.1	10.2	10.2	10.4	0.21	ns	ns
MCHC, g/dL	34.3^{x}	34.5^{x}	34.3^{x}	33.4^{y}	33.9^{xy}	33.9^{b}	33.8^{b}	34.5^{a}	34.3^{ab}	34.0^{ab}	0.83	**	**
PLT, x 10^3 cells/µL	813.5^{x}	796.1^{xy}	707.3^{z}	697.4^{z}	717.5^{yz}	720.0^{ab}	750.6^{ab}	753.5^{ab}	799.1^{a}	708.6^{b}	139.0	**	*

[a.] Treat.= Treatment;
Different letters mean significant differences;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
F= furfural; 2-HBA= 2-hydroxybenzaldehyde; 2-UND: 2-undecanone; ABZ: albendazole;
Time1: 1 days after F, 2-HBA, 2-UND and ABZ treatment; Time2: 3 days after F, 2-HBA, 2-UND and ABZ treatment; Time3: 7 days after F, 2-HBA, 2-UND and ABZ treatment, Time4: 14 days after F, 2-HBA, 2-UND and ABZ treatment; Time5: 21 days after F, 2-HBA, 2-UND and ABZ treatment;
RBC = Red Blood Cells, HGB = Haemoglobin, HCT = Hematocrit, MCV = Mean Corpuscular Volume of Red Blood Cells, MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration, PLT= Total Platelets.

Table 3. Total and differential white blood cells count of Sarda dairy ewes during the experiment.

	Time					Treatment						P-value	
Parameter	**1**	**2**	**3**	**4**	**5**	**Control**	**F**	**2-HBA**	**2-UND**	**ABZ**	**SEM**	**Time**	**Treat.[a]**
WBCB, x 10^3cells/µL	8.2^{xy}	8.7^{x}	7.5^{y}	7.4^{y}	8.2^{xy}	8.4^{a}	7.1^{b}	7.4^{b}	8.5^{a}	8.6^{a}	1.16	**	**
NEUTS, x 10^3cells/µL	41.5^{xy}	37.3^{yz}	37.4^{yz}	36.1^{z}	42.0^{x}	38.1^{ab}	39.8^{ab}	40.5^{a}	39.9^{ab}	35.8^{b}	6.20	**	*
LYMPHS, x 10^3cells/µL	47.2^{xy}	50.5^{x}	51.3^{x}	49.6^{x}	45.2^{y}	49.7	48.4	48.0	47.5	50.1	4.55	**	ns
MONOS, x 10^3cells/µL	3.9^{y}	3.9^{y}	3.6^{y}	5.9^{x}	4.9^{xy}	4.8	4.5	3.9	3.6	5.4	2.10	**	ns
EOS, x 10^3cells/µL	5.9	6.8	5.9	6.2	6.1	5.5^{ab}	5.2^{b}	5.9^{ab}	7.1^{a}	7.1^{a}	2.87	ns	**
BASOS, x 103 cells/µL	0.7^{y}	0.9^{xy}	1.0^{x}	0.9^{xy}	1.0^{x}	0.9^{b}	1.1^{a}	0.8^{b}	0.9^{b}	0.8^{b}	0.18	**	**

[a.] Treat.= Treatment;
Different letters mean significant differences;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
F= furfural; 2-HBA= 2-hydroxybenzaldehyde; 2-UND: 2-undecanone; ABZ: albendazole;
Time1: 1 days after F, 2-HBA, 2-UND and ABZ treatment; Time2: 3 days after F, 2-HBA, 2-UND and ABZ treatment; Time3: 7 days after F, 2-HBA, 2-UND and ABZ treatment, Time4: 14 days after F, 2-HBA, 2-UND and ABZ treatment; Time5: 21 days after F, 2-HBA, 2-UND and ABZ treatment;
WBCB = White Blood Cells, NEUTS = Neutrophil Cells, LYMPHS = Lymphocytes Cells, MONOS = Monocytes Cells, EOS = Eosinophils Cells, Basos = Basophils Cells.

Table 4. Means of faecal worm egg counts of Sarda dairy ewes during the experiment.

	Time			Treatment						P-value	
Parameter	**1**	**2**	**3**	**Control**	**F**	**2-HBA**	**2-UND**	**ABZ**	**SEM**	**Time[1]**	**Treatment[2]**
epg	2302[a]	1041[b]	1396[ab]	1891[a]	2431[a]	1489[ab]	2008[a]	77.1[b]	1563	*	**

Different letters mean significant differences;
ns = not significant with P>0.05;. *= P≤0.05; **= P≤0.01;
F= furfural; 2-HBA= 2-hydroxybenzaldehyde; 2-UND: 2-undecanone; ABZ: albendazole;
Time1: 7days after F, 2-HBA, 2-UND and ABZ treatment; Time2: 14 days after F, 2-HBA, 2-UND and ABZ treatment;Time3: 21 days after F, 2-HBA, 2-UND and ABZ treatment;
[1]Time*[2]Treatment= not significant.

Table 5. Regression equation, limit of detection and quantification for FA, SA, 5-HMF, F and 2-HBA by HPLC-DAD.

Compounds	Rt (min)	Regression equation	Linearity range (mg/L)	R^2	LOD (S/N=3) (mg/L)	LOQ (S/N=10) (mg/L)
2- furoic acid	2.2	y= 69.72x + 91.03	0.10 - 15	0.992	0.05	0.10
salicylic acid	2.2	y=41.07x - 7.176	0.10 - 15	0.999	0.10	0.20
5-hydroxymethylfurfural	2.7	y= 294.2x – 16.82	0.10 - 15	0.999	0.05	0.13
furfural	3.8	y= 360.5x – 26.4	0.10 - 15	0.999	0.05	0.10
2-hydroxybenzaldehyde	6.3	y= 149.4x + 0.206	0.10 - 15	0.999	0.05	0.10

Table 6. Regression equation, limit of detection and quantification for 2-UND by GC-MS.

Compounds	Rt (min)	Regression equation	Linearity range (mg/L)	R^2	LOD (S/N=3) (mg/L)	LOQ (S/N=10) (mg/L)
2-UND	16.0	y= $7e^6$x -68651	0.05 - 100	0.999	0.25	0.05

Table 7. Regression equation, limit of detection and quantification for ABZ-SO by LC-MS.

Compounds	Rt (min)	Regression equation	Linearity range (mg/L)	R^2	LOD (S/N=3) (mg/L)	LOQ (S/N=10) (mg/L)
ABZ-SO	11.6	y= $7e^6$x -68651	0.01 - 30	0.992	0.005	0.01

Figure 1. GC-MS chromatogram of 2-undecanone (R_t= 16.0 min.) in milk sample.

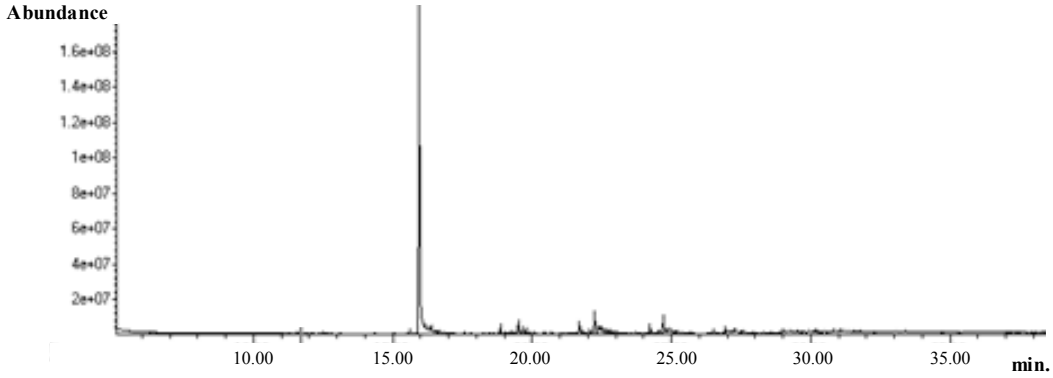


Figure 2. LC-MS/MS MRM chromatogram of ABZ-SO in milk sample. Peaks: 1 (unknown), 2 (ABZ SO), 3, 4 and 5 (unknown). For quantification of ABZ-SO we monitored the following transition with the corresponding energy (282.0>158.8: 36 V; 282.0>207.8: 24 V; 282.0>239.6: 16 V;).

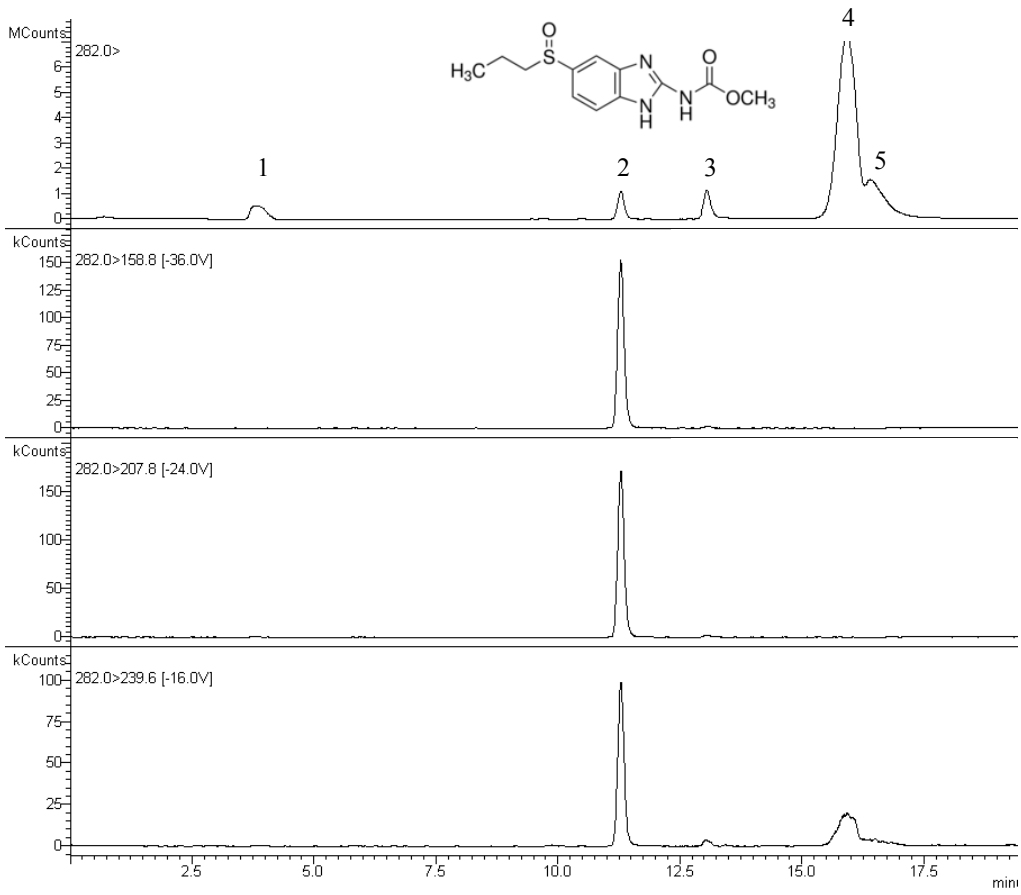
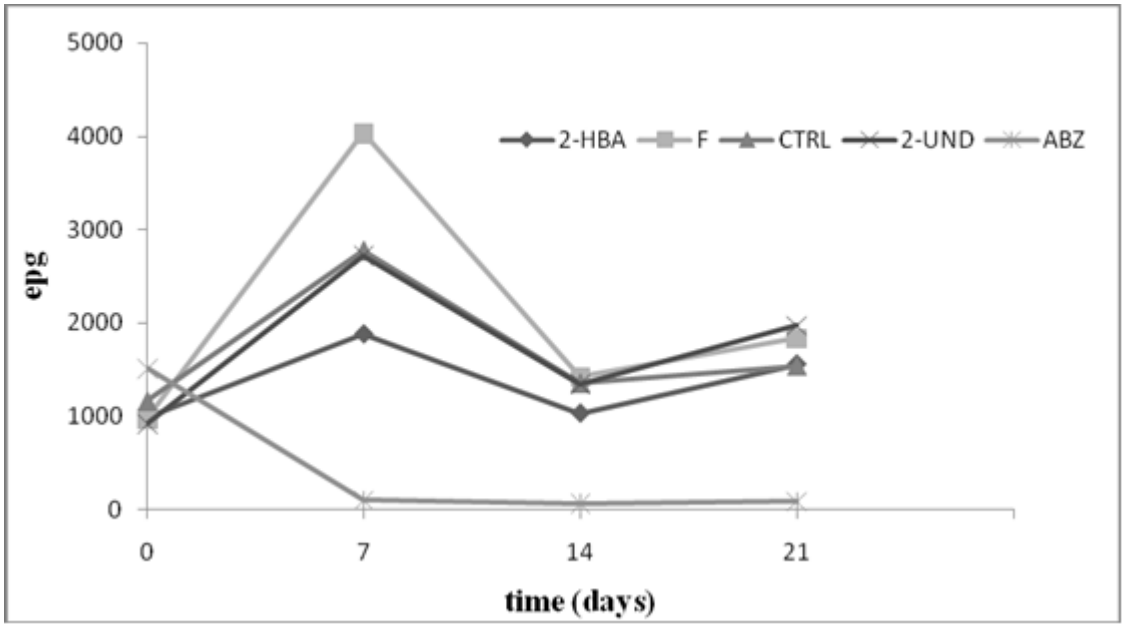


Figure 3. Eggs per gram mean of the five groups during the experimental trial.



2-HBA= 2-hydroxybenzaldehyde; F= furfural; CTRL= control; 2-UND: 2-undecanone;
ABZ: albendazole;

General conclusions

Results reported in this PhD thesis were:

- ✓ the *in vitro* studies showed clearly that *Ruta chalepensis* L. extracts possess anthelmintic activity against gastrointestinal nematodes, especially methanol extract showed the highest anthelmintic activity with an EC_{50}= 0.10 mg/mL after 96h treatment, while the *R. chalepensis* L. essential oil an EC_{50}= 1.20 mg/mL after 48h treatment;
- ✓ authentic standards of selected phytochemicals 2-undecanone and (E,E)-2,4-decadienal showed the highest nematicidal activity against gastrointestinal nematodes with a $EC_{50/24h}$= 0.88 and 1.03 mg/mL respectively, while for furfural, 2-hydroxybenzaldehyde and epigallocatechin gallate this concentration reference was 1.83 and 2.19 mg/mL at 24h and 1.79 mg/mL at 48h, respectively;
- ✓ in L_1 lungworms, the highest nematicidal activity was for 2-hydroxybenzaldehyde after 48 hours with an EC_{50} = 0.09 mg/mL, while furfural exhibited the highest activity after 24 hours with EC_{50}= 1.70 mg/mL;
- ✓ the *in vitro* experiment revealed dose-dependent anthelmintic activity;
- ✓ after administration of furfural and 2-hydroxybenzaldehyde in Sarda dairy ewes, the compounds and their metabolites were not detected in milk, urine and faecal samples;
- ✓ in *in vivo* experiments, ewe blood parameters were generally within the normal range but significant differences can be seen through different groups;
- ✓ using higher doses of furfural and 2-hydroxybenzaldehyde, the compounds and their metabolites were not detected in milk samples;
- ✓ 2-undecanone were no detected in milk samples;
- ✓ no carry over of these compounds was detected in milk.
- ✓ in the withdrawal interval albendazole sulfoxide was detected in milk 24 hours after treatment;
- ✓ the administration of furfural, 2-hydroxybenzaldehyde and 2-undecanone did not caused toxicological effects;

- ✓ no negative effects were observed in animals treated with furfural, 2-hydroxybenzaldehyde, 2-undecanone and albendazole;
- ✓ *in vivo* anthelmintic activity of furfural, 2-hydroxybenzaldehyde and 2-undecanone may be improved with different formulation and studying their mode of action and safety.