CHAPTER 12

THE USE OF ESSENTIAL OILS AGAINST SHEEP GASTROINTESTINAL NEMATODES

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INTRODUCTION

Gastrointestinal nematodes (GINs) nowadays present a serious threat to sustainable sheep breeding. Various classes of commercial anthelmintics were used to control these parasites. However, due to the development of anthelmintic resistance (AR), the efficacy of these drugs has decreased, which in turn resulted in high economic losses. For these reasons, researchers are focused now on designing sustainable strategies for GIN control, based on the use of a combination of a bunch of options including the wise application of anthelmintic drugs, as well as applying alternative strategies. Within this context, phytotherapy (the use of plants or their products) presents one of the most promising alternatives. Essential oils (EOs) are natural, volatile and complex compounds characterized by a strong odor and extracted from aromatic plants. In various studies so far, these plant products showed high in vitro and, in some cases, in vivo efficacy against sheep GINs. The aim of this chapter is to review so far conducted studies based on the use of EOs against these parasites and to discuss results, as well as advantages of their use compared to commercial anthelmintics. On the other hand, current obstacles in the use of EOs and possible solutions on how to overcome them will be also discussed in this chapter. In this way, current and future perspectives of the use of EOs against sheep GINs are discussed here.

Sheep Gastrointestinal Nematodes and the Problem of Anthelmintic Resistance

Infections caused by gastrointestinal nematodes (GINs) are currently considered as one of the main obstacles for breeders of grazing sheep worldwide (Hammer et al. 2019). Although these infections are most commonly subclinical, manifested as impaired weight gain and lowered milk yields but in some cases they can lead to serious conditions such as anaemia, diarrhea, digestive problems, protein loss, lowered immunity and fertility and even death (Giovanelli et al. 2018; Bosco et al. 2020; Belecké et al. 2021). Therefore, the negative effect of these parasites is reflected in various ways, from impaired animal health and welfare and reduced growth to a decrease in animal productivity and farm profitability (Velde et al. 2018). The economic losses caused by gastrointestinal parasitism are huge and difficult to estimate, although some reports indicate that these are estimated to be 17.94% of the total economic cost in animals (Abbas et al. 2020).

Nowadays, these parasites are widely distributed in many parts of the world. Generally, Haemonchus spp. and Cooperia spp. are more prevalent in sub-tropical/tropical environments, Ostertagia and Nematodirus spp. in the temperate regions, while Trichostrongylus spp. are prevalent throughout the world (Waller 2006). The prevalence of sheep GINs in Serbia is also high, with the following genera identified: Nematodirus spp. 71.22%, Ostertagia spp. 69.22%, Trichostrongylus spp. 66.55%, Haemonchus spp. 64.44% and Chabertia spp. 60.11% in Vojvodina, lowland landscape (Pavlović et al. 2017) as well as Haemonchus spp. (46.91%), Oesophagostomum spp. (40.73%), Trichostrongylus spp. (39.85%), Nematodirus spp. (35.88%) and Chabertia spp. (32.79%) in Eastern Serbia, predominantly mountainous (Kulišić et al. 2013). In southern Italy, the prevalence of sheep GIN genera varies but includes Haemonchus spp. (21-83%), Trichostrongylus spp. (2-59%), Chabertia spp. (0-48%), Teladorsagia spp. (0-25%) and Cooperia spp. (0-5%) (Bosco et al. 2020).

The control of sheep GINs is currently nearly exclusively reliant on commercial anthelmintic drugs (Bosco et al. 2020, Castagna et al. 2021). These include benzimidazoles (eg. albendazole, fenbendazole, mebendazole), macrocyclic lactones (eg. ivermectin, moxidectin, eprinomectin) and imidazothiazoles (eg. levamisole) (Dyary 2018; Velde et al. 2018). However, their improper use that refers to overfrequent treatments, miss-use or dose as well as continued use of one drug, has led to the development of anthelmintic resistance (AR) in different nematodes species and strains (Dyary 2018; Pinto et al. 2019; Bosco et al. 2020; Belecké et al. 2021), which is now reported worldwide. This has also been reported against even newly developed drugs such as monepantel (Mederos et al. 2014), whereby AR to a new drug has been reported in less than 10 years after introduction to the market. Furthermore, widespread incidence of multidrug-

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resistant populations of Haemonchus contortus, Teladorsagia and Trichostrongylus to benzimidazoles, imidazothiazoles and macrocyclic lactones in sheep throughout Europe has also been reported (Fissiha and Kinde 2021). Therefore, the reduced susceptibility of drugs in nematodes has resulted in even higher economic losses, which in turn endangers the sustainability of livestock (Vineer et al. 2020).

Sustainable Strategies for the Control of Sheep Gastrointestinal Nematodes

Modern sustainable strategies in sheep GINs management are based on rational use of anthelmintics along with the use of alternative strategies. Within the use of commercial anthelmintics, incorporation of refugia is suggested which is based on the treatment of only a proportion of animals instead of the whole group. In such conditions, one part of the parasite population remains untreated, which allows the maintenance of drug-sensitive parasites (Hodgkinson et al. 2019). The best-recommended practices for preserving refugia are targeted treatments (TT), that are related to the treatment of the whole flock based on knowledge of the risk or parameters that quantify the severity of infection as well as target selective treatments (TST), which are based on the treatment of only individual animals within the group to provide epidemiological and/or production benefits (Calvete et al. 2020). In essence, both TT and TST signify the rational use of drugs, i.e. on animals that require treatment due to health, productivity and welfare reasons, whereby single or combined pathophysiological and/or production-based treatment indicators are used for the selection of animals. These include clinical signs, faecal egg count (FEC), FAMACHA© scores, weight gain, milk production, body condition score (BCS), breech soiling and the diarrhoea score (DAG) (Soto-Barrientos et al. 2018; Calvete et al. 2020). Combining anthelmintics which have a related spectrum of activity but different modes of action, as well as the rotation of used anthelmintic classes, are also recommended as a way of slowing down the development of AR (Fissiha and Kinde 2021). On the other hand, these strategies could be complemented or even replaced with alternative solutions for controlling sheep GINs. Genetic control methods involve the selection of animals resistant to GIN, which as a process relies on the existence of genetic variation in the host and the predominant environmental conditions (Zvinorova et al. 2016). The objective of pasture management is to minimize the use of anthelmintics by avoiding exposure to parasite burden that would lead to clinical disease and loss of production, while simultaneously aiming to allow sheep to build up immunity (Abbott et al. 2012). Nutritional manipulation relies on a balanced grazing system that provides an adequate source of nutrients and an acceptable GIN burden, which in turn should allow an optimum level of productivity (Torres-Acosta et al. 2012). Biological control measures include the use of natural enemies against GINs, which mostly refers to different nematophagous fungal species such as Duddingtonia spp. (Szewc et al. 2021). In the end, the development of efficient vaccines against intestinal parasites will allow antiparasitic medications to be used less frequently (Fissiha and Kinde 2021).

Among the proposed alternative strategies for the control of sheep GINs, phytotherapy is considered particularly promising. Phytotherapy is defined as the use of plants to treat ailments, which as a healthcare practice is recorded since ancient times and transferred from generation to generation (Borges and Borges 2016; Castagna et al. 2021). In veterinary medicine, there is an increasing interest in validating ethnoveterinary practices, judging by the high number of studies and articles on the topic (Eshetu et al. 2015). Antiparasitic properties are a common point of focus, whereby a wide range of plants or their products are considered suitable for the treatment of almost every parasitic disease in livestock (Athanasiadou et al. 2007). For this purpose, whole plants (e.g. rich in tannins), their parts or products such as extracts (e.g. aqueous and ethanol) and essential oils may be used. Within this context, plant based antiparasitic preparations may provide successful alternative remedies to synthetic drugs and be used in ethnoveterinary practices against sheep GINs (Castagna et al. 2021).

Properties and Chemical Composition of Essential Oils

Essential oils (EOs) are natural, volatile and complex mixtures of compounds characterized by a strong odor and extracted from aromatic plants (Bakkali 2008). As secondary metabolites, they are present in the specialized cells or glands and serve to protect plants from predators and pests, while also attract pollinators. These cells are present in all sections of these plants including flowers, leaves, buds, stems, twigs, seeds, fruits, roots, wood or bark (Bakkali 2008; Butnariu and Sarac 2018; Fokou et al. 2020). Therefore, EOs are considered as part of the plant immune system (Butnariu and Sarac 2018). As far as physical and chemical properties are concerned, EOs are liquid, volatile, and rarely colored, lipid-soluble and soluble in organic solvents with a generally lower density than that of water. These plant products are mostly extracted from flowers and leaves of various aromatic plants growing in temperate and warm regions such as the Mediterranean, the Amazon or tropical countries, where they represent an important part of traditional pharmacopoeia (Bakkali 2008).

The medicinal properties of EOs are recognized since ancient times which include antiviral, antibacterial, antifungal, antiparasitic, anti-inflammatory, antiseptic, anticancer and antispasmodic properties among others (Bakkali 2008; Mancianti and Ebani 2020; Zaman et al. 2020). In veterinary medicine, EOs are increasingly used for the prevention and treatment of various animal diseases. Although this still mostly refers to monogastric animals such as pigs and poultry (Mucha and Witkowska 2021), some implications and reports suggest their possible use as anthelmintic agents in ruminants as well (André et al. 2018). EOs may be obtained from plants by different methods of extraction, but the most common industrial methods are steam distillation and extraction with different solvents (Butnariu and Sarac 2018).

The active ingredients of EOs are responsible for their pharmaceutical effects. To date, approximately 5000-7000 different constituents of EOs are identified and described in which mono- and sesquiterpenes predominated, along with aromatic compounds such as phenylpropane derivates (Morsy 2017; Butnariu and Sarac 2018) as mentioned in Table 1. Terpenes present polymers of isoprene (C_5H_8) and may be divided into hydrocarbons or oxygenated derivatives (oxides, alcohols, aldehydes, ketones, acids) or reaction products thereof (esters, ethers) (Butnariu and Sarac 2018). On the other hand, the group of phenylpropenes comprises constituents derived from n-propyl benzene (Morsy 2017). EOs have a very high variability of their composition, both in qualitative and quantitative terms (Dhifi et al. 2016).

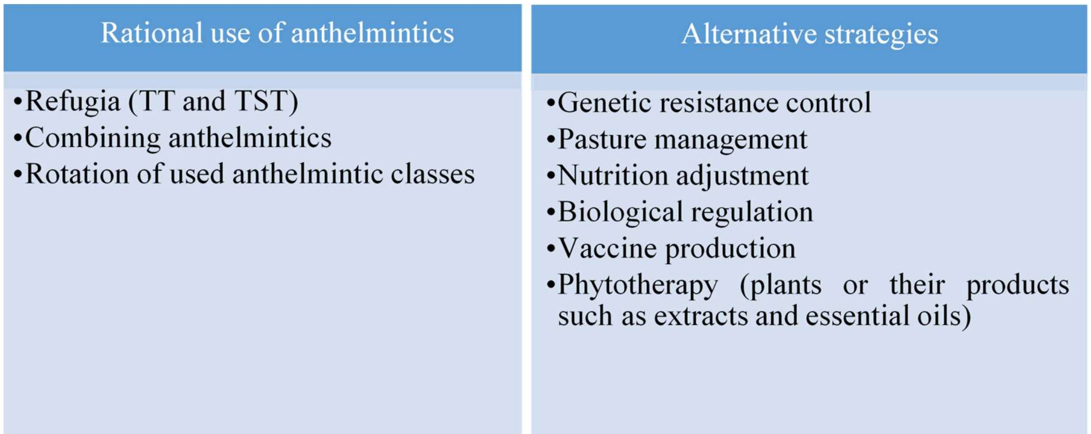

Fig. 1: Strategies for sustainable control of sheep GINs.

Table 1: Constituents of essential oils and their division by chemical groups and examples (Dhifi et al. 2016; Morsy 2017; Butnariu and Sarac 2018)

Chemical group	Examples
Hydrocarbon terpenes	Limonene, α- and β-pinene, camphene, α- and γ-terpinene, sabinene, myrcene, β-caryophyllene, germacrene B and D, o and p-cymene etc.
Oxygenated derivates (terpenoids)	a) Phenols - thymol, carvacrol
	b) Alcohols - linalool, citronellol, geraniol, menthol, α-terpineol, terpinen-4-ol, borneol
	c) Aldehydes - citral, citronellal, sinensal
	d) Ketones - α i β-tujon, camphor (2-bornenon), menton, carvone
	e) Oxydes - eucalyptol (1,8-cineole)
	f) Esters - linalyl-acetate, geraniol-acetate,
Phenylpropenes	Anethole, methyl chavicol (estragole), eugenol, vanillin, safrole, myristicin, cinnamaldehyde
Miscellaneous (sulfur- and nitrogen-containing compounds)	allyl sulfide, allicin, methyl anthranilate, indole, pyridine, pyrazine

Many factors such as light, precipitation, growing site (altitude, latitude), nature of the soil (pH, constituents), site of production and accumulation of the EOs in the plant, the age of the plant, the presence of soil organisms and microorganisms, predators and pollinators as well as postharvest treatment of EOs (Barra 2009; Fokou et al. 2020) may affect their chemical composition, which ultimately lead to variation in their pharmacological properties.

The Potential use of Essential Oils against Sheep Gastrointestinal Nematodes

In Vitro Tests

The interest of the use of EOs against sheep gastrointestinal nematodes, as well the number of studies upon that are in increasing trend over years. Within that context, different EOs showed anthelmintic potential so far. In vitro tests present the first step in the process of validating phytotherapy substances and are used for the initial evaluation and selection of plant species and their secondary metabolites that exhibit anthelmintic activity (Borges and Borges 2016; André et al. 2017; Štrbac et al. 2021a). Among in vitro tests, the most reliable and most common used tests are egg hatch assay (EHA) and larval development assay (LDA) that reflect ovicidal and larvicidal potential of EOs, as well as different larval and adult motility assays that suggest the effect of EOs on the motility of larva's or adults (Table 2). The advantages of the use of in vitro tests are ease of application, low cost, speedy, high reproducibility and no need for experimental animals (protection of animal welfare), and thus have been widely used in the screening of medicinal plants, often rather than in vivo tests (Ferreira et al. 2016).

EOs listed in Table 2. showed anthelmintic potential against sheep GINs (mostly against H. contortus), but it differed depending on the oil used. The highest ovicidal activity, expressed as IC50 values, was recorded for Cymbopogon schoenanthus and Cymbopogon martinii (0.04 and 0.1 mg/mL, respectively, Katiki et al. 2011), Thymus vulgaris (0.098 mg/mL, Štrbac et al. 2021a), Ruta chalepensis (0.1 mg/mL, Akkari et al. 2015), and Mentha arvensis (0.1 mg/mL, Chagas et al. 2018). Different EOs of Lippia spp. showed great larvicidal activity with IC50 less than 0.01 mg/mL (Chagas et al. 2018), followed by Thymus vulgaris with IC50 as 0.062 mg/mL (Ferreira et al. 2016), Hesperozygis myrtoides with IC50 as 0.07 mg/mL (Castilho et al. 2017), Piper aduncum with IC50 as 0.1 mg/mL (Gaínza et al. 2016) and Mentha piperita with IC50 as 0.2 mg/mL (Katiki et al. 2011). Cymbopogon schoenanthus exhibited a very high activity on the larval motility with IC50 as 0.009 mg/mL (Katiki et al. 2012), whereby Ruta chalepensis induced 87.5% inhibition of motility of adults 8 h after exposure at the dose of 1 mg/mL (Akkari et al. 2015). Along with Thymus vulgaris, Origanum vulgare, Foeniculum vulgare and Satureja montana showed a high ovicidal effect in our studies with inhibition of egg hatchability up to 100% for each concentration tested, 0.049-50 mg/mL (Štrbac et al. 2021a; Štrbac et al. 2022). In certain experiments, bioactive compounds of EOs are also evaluated for anthelmintic activity, mostly against H. contortus and with the same tests. The list is also wide and includes anethole, B-elemene, borneol, camphor, carvacrol, carvone,

Table 2: Essential oils that have shown *in vitro* activity against sheep gastrointestinal nematodes, assays and references

Essential oil(s)	Assays	GIN species used	Reference
Croton Zehtneri (two samples)*, Lippia sidoides*	EHA, LDA	*Haemonchus contortus*	Camurça-Vasconcelos et al. 2007
Eucalyptus globulus	EHA, LDA	*H. contortus*	Macedo et al. 2009
Cymbopogon schoenanthus, Cymbopogon martinii, Mentha piperita	EHA, LDA, LFIA, LEA	*H. contortus and Trichostrongylus spp*	Katiki et al. 2011
Arisaema lobatum, Arisaema franchetianum	EHA, LDA, LMIA	*H. contortus*	Zhu et al. 2013a
Artemisia lancea	EHA, LDA, LMIA	*H. contortus*	Zhu et al. 2013b
Tagetes minuta, Coriandrum sativum Alpinia zerumbet, Lantana camara	EHA, LDA	*H. contortus*	Macedo et al. 2013
Eucalyptus citriodra	EHA, LDA	*H. contortus*	Ribeiro et al. 2014
Melaleuca alternifolia	EHA, LMIA	*H. contortus*	Grando et al. 2015
Zanthoxylum simulans	EHA, LDA, LMIA	*H. contortus*	Qi et al. 2015
Cymbopogon citratus	EHA, LDA	*H. contortus*	Macedo et al. 2015
Ruta chalepensis	EHA, AWMA	*H. contortus*	Akkari et al. 2015
Citrus sinensis, Melaleuca quinquenervia	EHA, LDA	*H. contortus*	Gaínza et al. 2015
Thymus vulgaris	EHA, LDA, LMIA, AWMA	*H. contortus*	Ferreira et al. 2016
Piper aduncum	EHA, LDA	*H. contortus*	Gaínza et al. 2016
Hesperozygis myrtoides	EHA, LDA	*H. contortus*	Castilho et al. 2017
Lavandula officinalis, Citrus aurantifolia, Anthemis nobile	EHA, LDA, AWMA	*H. contortus*	Ferreira et al. 2018
Mentha arvensis, Zingiber officinale, Lippia sidodes, Lippia alba, Lippia origanoides, Lippia gracilis, Curcuma longa, Mentha piperita	EHA, LDA	*H. contortus*	Chagas et al. 2018
Rosmarinus officinalis	EHA, LMIA	natural-mixed infection	Pinto et al. 2019
Eucalyptus citriodra	AWMA	*H. contortus*	de Araújo-Filho et al. 2019
Origanum majorana	EHA, AWMA	*H. contortus*	Abidi et al. 2020
Juniperus communis	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2020a
Coriandrum sativum	LMIA	H. contortus, *Trichostrongylus axei*, *T. colubriformis*, *T. vitrinus Teladorsagia circumcincta*, and *Cooperia oncophora*	Helal et al. 2020
Achillea millefolium, two chemotypes	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2020b
Origanum vulgare, Satureja hortensis, Thymus vulgaris, Mentha piperita, Helichrysum arenarium	EHA	natural-mixed infection: *Haemonchus* spp, *Trichostrongylus* spp, *Teladorsagia* spp and *Chabertia* spp	Štrbac et al. 2021a
Cinnamomum verum, Syzygium aromaticum	Mortality of nematode larvae in plant oil solution	*H. contortus*	Boyko and Brygadyrenko 2021
Cinnamomum verum, Syzygium aromaticum, Melaleuca alternifolia, Piper cubeba, Citrus aurantiifolia, Lavandula angustifolia	Mortality of nematode larvae in plant oil solution	*S. papillosus*	Boyko and Brygadyrenko 2021
Ocimum basilicum, 16 cultivares	EHA	*H. contortus*	Sousa et al. 2021
Origanum vulgare, Pimienta dioica	EHA, larval mobility	*H. contortus* and *Cooperia* spp.	Jiménez-Penago et al. 2021

**EHA - egg hatch assay; LDA - larval development assay; LMIA - larval motility inhibition assay; AWMA - adult worm motility assay, LFIA - larval feeding inhibition assay, LEA - larval exsheathment assay*

citral, cinnamaldehyde, eucalyptol, eugenol, linalool, thymol, terpinen-4-ol and vanillin among the others (Katiki et al. 2017; André et al. 2018). In a study of Katiki et al. (2017), the highest ovicidal activity was shown by cinnamaldehyde, anethole, carvone, carvacrol and thymol with IC50 of 0.018, 0.07, 0.085, 0.11 and 0.13 mg/mL, respectively. The high larvicidal effect of carvacrol and thymol was demonstrated in studies of André et al. (2016) and Ferreira et al. (2016) with IC50 values of 0.2 and 0.06 mg/mL, respectively. The activity of these phenolic compounds may be associated with damage caused to the cuticle and digestive apparatus on nematode larva's and neurotoxic effects on the free-living nematodes (interaction with SER-2 tyramine receptor) (André et al. 2016; 2017). Citral was also one of the most potent EO ingredients with an IC50 value of 0,13 mg/mL in EHA (Macedo et al. 2015).

In some cases, the efficacy of binary, ternary and quaternary combination of EO isolated compounds was evaluated as well, whereby the highest ovicidal activities were shown by cinnamaldehyde:carvacrol (1:1), anethole:carvone (1:1) and anethole + carvone + cinnamaldehyde + carvone (1:1:1:1) with

Table 3: *In vivo* efficacy of essential oils against sheep gastrointestinal nematodes

Essential oil	Test, the time of evaluation and GIN species	Dose, routes of administration and duration of use	Efficacy	Reference
Lippia sidoides	FECRT; Days 0, 7, 14 and 21 a.t.	230 mg/Kg, oral, during 5 days 283 mg/Kg oral, during 5 days	38.0% at D7; 30.0% at D14; 29.8% at D21 45.9 at D7;-54.0% at D14; 22.9% at D21	Camurça-Vasconcelos et al. 2008
Lippia sidoides	Controlled test; Day 7 a.t.; *Haemonchus* spp. and *Trichostrongylus* spp.	283 mg/Kg, oral, during 5 days	*Haemonchus spp.* 56.9% *Trichostrongylus* spp. 39.3%	Camurça-Vasconcelos et al. 2008
Orange oil emulsion	FECRT; Days 0 and 14 a.t.; *H. contortus*	600 mg/Kg, single 600 mg/Kg during 3 days	97.4% 94.9%	Squires et al. 2010
Cymbopogon schoenanthus	FECRT; Days 0, 1, 5, 10, 15 and 20 a.t.; *H. contortus*	180 and 360 mg/Kg during 3 days, oral	n.e.	Katiki et al. 2012
Cymbopogon schoenanthus	Controlled test; Day 20 a.t.; *H. contortus*	180 and 360 mg/Kg during 3 days, oral	n.e.	Katiki et al. 2012
Eucalyptus citriodora	FECRT; Days 0,10 and 17 a.t.; *Haemonchus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp.	250 mg/Kg	55.9% at day 10; 34.5% at day 17	Ribeiro et al. 2014
Thymus vulgaris	FECRT; Days 0, 2, 4, 6, 8, 10, 12 a.t.; *H. contortus*	75, 150 and 300 mg/Kg oral on Days 0, 6 and 12 a.t.	n.e.	Ferreira et al. 2016
Mentha arvensis	FECRT; Days 0, 1, 3, 7, 14 and 21 a.t.; *H. contortus* and *Trichostrongylus* spp.	200 mg/Kg, single dose	61.6% at D1; 48.1% at D14; 44.9% at D21	Chagas et al. 2018
Cymbopogon citratus	FECRT; Days 0, 8 and 15 a.t.; *Haemonchus spp, Trichostrongylus spp and Oesophagostomum spp.*	500 mg/Kg, oral, during 3 days	19.6% at D8; 23.9% at D15	Macedo et al. 2019
Cymbopogon citratus	Controlled test; Day 15 a.t. *H. contortus, T. colubriformis, O. columbianum, T. ovis*	500 mg/Kg, oral, during 3 days	*H. contortus* 66.4% *T. colubriformis* 38.4%	Macedo et al. 2019
Eucalyptus citriodora	FECRT, Days 0, 7 and 14 a.t. *Haemonchus* spp. *Trichostrongylus* spp., *Oesophagostomum* spp. and *Strongyloides* spp.	500 mg/Kg, oral, single dose	41.8% at D7; 69.5% at D14	de Araújo-Filho et al. 2019
Thymus vulgaris	FECRT; Days 0, 7 and 14 a.t.; *Haemonchus* spp.; *Trichostrongylus* spp.; *Teladorsagia* spp.; *Chabertia* spp.	100 mg/Kg, oral, single dose	25.23% at D7; 24.42% at D14	Štrbac et al. 2021b

** FECRT - faecal egg count reduction test; a.t. - after treatment; D - certain day after treatment; n.e. - not effective.*

IC50 values of 0.012, 0.013 and 0.02 mg/mL, respectively (Katiki et al. 2017). In our study, the activity of linalool:estragole binary combination at a ratio 19%:81% exhibited ovicidal activity with IC50 of 0.98 mg/mL (Štrbac et al. 2021c). However, as many studies have demonstrated so far, an EO often shows higher anthelmintic activity in comparison with the single isolated compound, due to the synergistic effect among many different constituents of the whole EO, although it should be stressed that a wide number of compounds is not crucial for high efficacy (Štrbac et al. 2022).

In Vivo Tests

The results obtained through in vitro tests must be confirmed in field condition trials. For this purpose, various in vivo studies are used to obtain the most authentic results of the efficacy of plant-based formulations (Table 3). Although these studies can be intensive, expensive and require time and animals for testing, these are essential as a further step in developing anthelmintic agents as they offer a clear picture of the possibility of using EOs and their ingredients against sheep GINs in everyday clinical practice. The most commonly used in vivo test is the faecal egg count reduction test (FECRT) which measures the percentage reduction in the number of nematode eggs excreted through faeces after administration of an active substance, and is confirmed by the controlled test that is based on the quantification of the worm burden after sacrificing animals which have previously been artificially inoculated with nematodes and treated (Kebede 2019).

As shown in Table 3, various EOs were found effective in different in vivo studies, whereby some were highly effective and some did not show any effect. Sometimes differences were found in efficacy of EOs of the same plant in different studies. This may be attributed to differences in chemical composition owing to variation in climate parameters, harvesting time, plant parts used, solvents used for extraction etc. Thus, EO of Thymus vulgaris showed some anthelmintic effects in our study (Štrbac et al. 2021b), although it failed to reduce FEC of GINs in a study of Ferreira et al. (2016) at even greater doses. Those differences may be related to the different compositions and the isolate of EO used, which was confirmed in our study with in vitro tested Achillea millefolium EO (Štrbac et al. 2020b) or to the even other factors. The dependence of EO efficacy on the method of application (single or multiple uses) was also contradictory.

The in vivo efficacy of the isolated EO compounds or their combination was also evaluated in some cases. Some of them showed a high effect on the reduction of FEC, such as carvacrol-acetate and thymol-acetate with the efficacy of 65.9 % and 76.2 % on Day 14 a.t., respectively (doses of 250 mg/kG) (André et al. 2016; 2017). In a study of Chagas et al. (2018), pure menthol, at the dose of 160 mg/Kg, did not express in vivo efficacy unlike the whole oil whose main ingredient is, i.e. Mentha arvensis that reduced FEC by approximately 50% on Days 1, 7 and 14 at a similar dose tested, 200 mg/Kg. In our study, the efficacy of the binary combination of linalool: estragole (19:81%) in the FECRT at the single dose of 100 mg/kG was evaluated, whereby efficacy was found to be 24.21% and 25.90% on Days 7 and 14, respectively (Štrbac et al. 2021b).

Toxicity Studies

Rarely, toxicity studies of the use of EOs or their ingredients against sheep gastrointestinal nematodes have been conducted. In two studies on mice, LD50 values determined for carvacrol and thymol were 919 mg/Kg and 1350.9 mg/Kg, whereby for their acetylated derivates carvacrol acetate (CA) and thymol acetate (TA), these values were 1544.4 mg/Kg and 4144.4 mg/Kg with no changes observed in the mice behavior (André et al. 2016; 2017). According to the guidelines proposed by Clark and Clarke (1977), orally administered substances with an LD50 value above 1000 mg/Kg are safe or considered as low-level toxic substances. So, CA, thymol and TA can be considered as non-toxic, while further studies should be performed for carvacrol. In a study of Ribeiro et al. (2014), the EO of Eucalyptus citriodora was classified as safe with an LD50 value of 2653.0 mg/Kg for mice. Some EO compounds such as menthol are of very low acute oral toxicity (LD50 > 2000 mg/Kg) (Chagas et al. 2018). Oral administration of the EO of Origanum majorana at different doses of 1000-5000 mg/Kg displayed no signs of toxicity, nor caused fatal effects in any of the treated mice during an observation period of 24 hours (Abidi et al. 2020). Katiki et al. (2012) concluded that Cymbopogon schoenanthus is safe for sheep at the doses of 180 mg/Kg and 360 mg/Kg, since no significant differences among group means for the hepatic (enzymes) or kidney (urea and creatinine) parameters were recorded after treatment with EO. In our in vivo studies, no toxic effects were observed on sheep, neither after oral administration of Thymus vulgaris (100 mg/mL) nor linalol:estragole (100 mg/mL) (Štrbac et al. 2021b).

Advantages and the Barriers of the use of Essential Oils to Control of Gastrointestinal Nematodes in Sheep

To date, EOs from various plants have shown efficacy against sheep GINs. As discussed above, their high anthelmintic potential is owed to various compounds that make up their composition, of which the primary component is most important (Dhifi et al. 2016). These compounds belong to different chemical groups, which impart antiparasitic activity through different mechanisms of action and synergism. These involve interruption of the nematode nervous system, interference with the neuromodulator octopamine or GABA-gated chloride channels, the inhibition of AChE activity, disruption of the cell membrane of the nematode thereby changing its permeability, membrane and ion channel perturbations modifying membrane-bound protein activity and the intracellular signaling pathways inducing different neurological and structural changes leading to nematode paralysis and death (Andrés et al. 2012). Apart from the high efficacy, different chemical origins of their ingredients may contribute to less susceptibility of EOs to anthelmintic resistance (Macedo et al. 2010; Borges and Borges 2016).

Moreover, the natural origin of plant-based formulations may contribute to less toxicity to hosts, as well as to fewer residues in meat and milk compared to synthetic drugs (Ferreira et al. 2018). Although this still needs to be confirmed, natural-based drugs are certainly much more environmentally acceptable. Finally, the use of chemical drugs is less and less sustainable not only due to AR, yet from the financial aspects, as drug prices continue to rise (Prakash et al. 2021). Also from that point of view, the use of different plant formulations could be a more sustainable and acceptable option given their low prices and ease of acquisition, especially in countries with developed biodiversity (Ferreira et al. 2018).

The main barriers in the use of EOs against sheep GINs as the widespread practice may be the lack of scientific data and trials aimed at verifying their efficacy against these parasites, which especially refers to in vivo trials. As discussed earlier, efficacy in field conditions must be proven before the use of some active substances in practice. Furthermore, toxicity studies should be conducted on the host animals. However, this field is relatively new and there is an increasing number of various studies aimed to confirm the efficacy and sustainable use of EOs against sheep GINs (Muthee 2018). Worsening of the situation due to AR forced many researchers worldwide to search for effective antiparasitic herbal formulations as a promising alternative to synthetic drugs. Our research group is actively engaged in the evaluation of new EOs for any anthelmintic efficacy through in vitro, in vivo and toxicity studies (data not shown).

The second problem about the current potential use of EOs in the practice is related to the low efficacy shown in field condition trials, which is still not comparable to commercially available anthelmintics (Macedo et al. 2010). Low in vivo efficacy is attributed to less bioavailability of active ingredients of EOs. This fact may be explained on the one hand by the anatomical and physiological specificity of the ruminant gastrointestinal tract (Hoste et al. 2008), and on the other hand by the unstable nature of EOs (Maes et al. 2019). Active ingredients of EOs are prone to evaporation and reaction with various factors inside the gastrointestinal tract. That leads to their partial or total inactivation before reaching the target place in abomasum or intestine. Thus, they usually show lower anthelmintic activity compared to that showed in different in vitro studies. Keeping in mind these factors, finding of plant species, the dose and route of administration effective in vivo is a challenge for ethnobotanists to be addressed. However, it also seems that increasing interest and the number of studies within this topic can contribute to overcoming this problem. Nevertheless, so far showed efficacy in different in vivo studies suggest that EOs and their active ingredients may be used as a valuable additional source in a nematode control along with other measures, if not capable to be used independently (Macedo et al. 2010).

Encapsulation as a Novel Approach

Encapsulation is the method of protecting active components via physical or chemical processes. In this way, the active substance is physically separated from the environment by the creation of a protective shell, often referred to as the active carrier component or matrix (Lević et al. 2014). Nowadays different encapsulation techniques are used such as emulsification, nanoprecipitation and coacervation with chitosan, alginate or cyclodextrin as matrices (Maes et al. 2019). Given the instability and volatility of EOs, encapsulation could be of great importance when it comes to their application against GINs in sheep. Encapsulation reduces the interaction of the active substances with various factors in the environment (Radunz et al. 2018) and leads to reduced inactivation of active ingredients of EOs in the animal which ultimately results in increased bioavailability. Also, encapsulation represents a sustainable and efficient approach to increasing physical stability and protection against evaporation, enabling longer retaining properties and shelf life of EOs (Majeed et al. 2015). The other advantages of encapsulation include the increase the ease of handling active substances, reduction of odor and unpleasant taste (may be important for oral administration), as well as controlled release of the active substance (Radunz et al. 2018).

In a study of Mesquita et al. (2013), emulsified EO of (italic) given orally to the sheep at the dose of 365 mg/Kg once, reduced the total number of nematodes in sheep gastrointestinal tract by 60.79%, which was better than ivermectin that reduced the number up to 48.70%. The reduction of abomasal nematodes was significantly higher in the group treated with EO (83.75% and 35.00%, respectively). Similarly, the nanoemulsion of the same oil at the dose of 250 mg/Kg once, reduced the FEC of GINs similarly to levamisole ($p>0.05$) in 8 of 10 days observed (Ribeiro et al. 2017). The dose of 250 mg/Kg of the encapsulated formulation of anethole:carvone (10% each and 80% of lipid matrix) given in food to lambs for 45 days significantly reduced FEC at the days 43 and 45, whereby the effect was attributed to a decrease in the size of males and a decrease in the fecundity of female nematodes (Katiki et al. 2019). At the same time, the formulation did not affect liver or kidney function of the lambs. When compared to free EO, nanoencapsulated oil of E. citriodora showed higher ovicidal (0.5 compared to 1.3 mg/mL) and similar larvicidal (both 1.7 mg/mL) in vitro, but slightly lower in vivo effect measured through faecal egg count reduction test at the same dose of 250 mg/Kg (40.5% compared to 55.9% at Day 10 a.t.) (Ribeiro et al. 2014). However, nanoemulsion of C. citratus showed clearly higher in vivo effect in the reduction of FEC compared to free EO, given orally for three days at the doses 450 and 500 mg/Kg, respectively (51.7% to 19.6% at Day 8 a.t.), whereby at the same time exhibited lower toxicity (Macedo et al. 2019). In most of these studies, chitosan was used as a carrier. However, studies aimed to confirm the positive impact of encapsulation techniques for the use of EOs against sheep GINs are needed.

Conclusion

In the era of AR, novel strategies for sustainable control of GINs in sheep farms should be designed. The use of EOs as an alternative method show great potential due to their high efficacy originating from rich chemical composition, their affordable price and easy acquisition, especially in countries with developed biodiversity. Along with this, EOs possess significantly less susceptibility to resistance and better host and environmental acceptability from the toxicity aspect in comparison with synthetic drugs. The major obstacles are reflected in the lack of trials conducted in field conditions, as well as still usually lower in vivo effects than commercial drugs. However, these obstacles may be overcome with an increasing number of field studies in different conditions, especially with EOs and their ingredients that showed great in vitro potential. Moreover, applying novel methods such as encapsulation offers an opportunity to protect active EO ingredients from degradation and inactivation and thus allow a higher in vivo efficacy. From all the above, the use of these plant products may significantly contribute to the sustainable control of sheep GINs in the near future.

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