MARTA MARGARIDA DE FIGUEIREDO OLIVEIRA

UNLOCKING THE POTENTIAL OF EXTREMOPHILE PLANTS FROM MARITIME ENVIRONMENTS AS SOURCES OF INNOVATIVE PRODUCTS FOR USE IN VETERINARY SCIENCES

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Doutoramento em Ciências Agrárias e Ambientais

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Marta Margarida de Figueiredo Oliveira)

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''...E assim, lição por lição
Que a pouco e pouco aprendemos
De outros – a outros daremos
Que a muitos outros darão''

António Aleixo

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SCIENTIFIC DISSEMINATION

Peer-reviewed articles in international journals in the scope of this thesis

- Oliveira, M., Dumouchel,A., Masson, L., Barbot, E., Pardo, E., Llorent-Martinez, E., Custódio, L. Hoste, H. *In vivo* anthelmintic effects of polyphenol-rich extracts from the salt-tolerant species *Cladium mariscus* L. Pohl and *Limoniastrum monopetalum* L. against gastrointestinal nematodes in lambs. *Submitted*.

- Oliveira, M., Pereira, C., Castañeda-Loaiza, V., Rodrigues, M.J., Neng, N.R., Hoste, H., Ben Hamed, K., Custódio, L. Seasonal biochemical variations in Mediterranean salt-tolerant plants for sustainable nutraceutical and phytotherapeutic innovations in ruminant health. *Submitted.*

- Oliveira, M., Sprengel Lima, C., Llorent-Martínez, E.J., Hoste, H., Custódio, L. (2022). Impact of seasonal and organ-related fluctuations on the anthelmintic properties and chemical profile of *Cladium mariscus* (L.) Pohl extracts. *Front. Plant Sci*. *13*, 934644. https://doi.org/10.3389/fpls.2022.934644

- Oliveira, M., Sprengel Lima, C., Ketavong, S., Llorent-Martínez, E.J., Hoste, H., Custódio, L. (2021). Disclosing the bioactive metabolites involved in the *in vitro* anthelmintic effects of salt-tolerant plants through a combined approach using PVPP and HPLC-ESI-MS^n. *Sci. Rep*. 11, 24303. https://doi.org/10.1038/s41598-021-03472-9

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- Oliveira, M., Hoste, H., Custódio, L. (2021). A systematic review on the ethnoveterinary uses of Mediterranean salt-tolerant plants: exploring its potential use as fodder, nutraceuticals or phytotherapeutics in ruminant production. *J. Ethnopharmacol. 267,* 113464. https://doi.org/10.1016/j.jep.2020.113464

Peer-reviewed articles in international journals outside the scope of this thesis

- Rodrigues, M.J., Pereira, C.G., Oliveira, M., Zengin, G., Custódio, L. (2023). Salt tolerant plants as sources of antiparasitic agents for human use: A comprehensive review. *Mar. Drugs*. *21* (2), 66. https://doi.org/10.3390/md21020066

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Oral and panel communications in international conferences

- Oliveira, M., Sprengel Lima, C., Ketavong, S., Llorent- Martínez, E., Hoste, H., Custódio, L. (2021, July 19-22). *Unravelling the potential bioactive metabolites linked to the in vitro anthelmintic properties of selected Mediterranean salt-tolerant plants* [**Oral presentation**]. 28th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Virtual Edition.

- Oliveira, M., Sprengel Lima, C., Ketavong, S., Llorent-Martínez, E., Hoste, H., Custódio, L. (2021, July 6-8). *The halophyte plant* Limoniastrum monopetalum *L. as source of anthelmintic metabolites targetting ruminants* [**Panel presentation**]. 4th Edition of International Summer School on Natural Products, Virtual Edition. ISSNP Third Prize in Poster Competition Award.

- Oliveira, M., Sprengel Lima, C., Ketavong, S., Hoste, H., Custódio, L. (2021, March 15-17). *In vitro anthelmintic properties of Mediterranean salt-tolerant plants against* Haemonchus contortus *and* Trichostrongylus colubriformis *larvae and eggs and investigation of its bioactive compounds* [**Panel presentation**]. 29th Annual Meeting of the German Society for Parasitology, Virtual Edition.

- Oliveira, M., Rodrigues, M., Pereira, C., Neng, N., Ketavong, S., Sprengel Lima, C., Hoste, H., Custódio, L. (2019, June 19-22). *A first insight into the nutritional value, phenolic content and biological activities of the halophyte* Cladium mariscus L. *Pohl* [**Oral presentation**]. Trends in Natural Product Research - PSE Young Scientists' Meeting on Biochemistry, Molecular Aspects and Pharmacology of Bioactive Natural Products, Budapest, Hungary.

- Oliveira, M.**,** Hoste, H., Custódio, L. (2019, May 29 - June 01). *Mediterranean halophytes and its potential use in animal nutrition and health: from ethnoveterinary uses to bioactive properties* [**Panel presentation**]. 4th International Conference on Natural Products Utilization - From Plants to Pharmacy Shelf, Albena, Bulgaria.

- Oliveira, M., Sprengel Lima, C., Ketavong, S., Cristofolli, V., Fabre, N.. Custódio L, Hoste, H. (2019, March 14-15). In vitro *anthelmintic effects of some Mediterranean halophyte plants against two gastrointestinal nematodes* [**Oral presentation**]. Haemonchus contortus *and* Trichostrongylus colubriformis .9èmes Journées du Consortium anti-Parasitaire et anti-Fongique (CaPF), Rouen, France.

- Oliveira, M., Rodrigues, M.J., Pereira, C., Santos, T., Barreira, L., Hoste, H., Custódio, L. (2017, September 17-20). *Potential of selected halophytes from Southern Portugal as sources of nutraceuticals with veterinary applications: preliminary screening of their phenolics contents and antioxidant properties* [**Panel presentation**]. PSE International Symposium, New & Old Phytochemicals: Their Role in Ecology, Veterinary & Welfare, Chieti, Italy.

THESIS OVERVIEW

This dissertation is divided in six chapters. Chapter I initiates with an overview on plant metabolites, with focus on phenolics and its applications for ruminant animals, narrowing to gastrointestinal nematodes infections (GIN) and its control and management strategies. Then, a bibliographic revision was conducted on salt-tolerant plants and its uses on animal traditional practices along the Mediterranean area and, by the end of this chapter, a summary on the selected salt-tolerant species to be included in this work is given. Chapters II-V are presented in the form of research articles, either submitted or published. In Chapter II, the performed work focuses on the screening of the nutraceutical and phytotherapeutic value of each selected species, considering seasonality. Findings of this chapter validated the number of samples to proceed for the anthelmintic experiments (Chapters III-V). In the following chapters, the *in vitro* anthelmintic evaluation of selected samples (Chapter III) was complemented by a detailed analysis on the season- and organ-related variations on the anthelmintic effects of one of the most active species, namely *Cladium mariscus* L. Pohl (Chapter IV). To sum up the research work, Chapter V deals with the *in vivo* anthelmintic outcomes of the two most promising species, namely *C. mariscus* and *L. monopetalum*, based on the *in vitro* results priorly achieved. Ultimately, Chapter VI aims at a cohesive discussion of the work developed throughout the thesis culminating on the main conclusions and future perspectives foreseen.

ABSTRACT

Mediterranean salt-tolerant plants, adapted to challenging conditions, are underlooked as sustainable anthelmintic solutions for ruminants, offering potential for livestock management, particularly in areas where saline or degraded soils restrict traditional agricultural productivity. With this in mind, the main goal of this dissertation is to valorize Mediterranean salt-tolerant plant species for the management of gastrointestinal nematode (GIN) infections in ruminants, either as nutraceutical plants or phytotherapeutic options. Target species were *Pistacia lentiscus* L., *Helichrysum italicum* subsp. *picardii* (Boiss. & Reut.) Franco, *Inula crithmoides* L.*, Calystegia soldanela* (L.) R.Br.*, Cladium mariscus* (L.) Pohl*, Medicago marina* L.*, Plantago coronopus* L.*, Limoniastrum monopetalum* L. Boiss, and *Crucianella marítima* L.. Chemical analyses disclose that these species are rich in minerals and phenolics, valued for their antioxidant properties, making them promising for nutraceutical and phytotherapeutic applications. Exploring seasonal variations allowed the selection of samples with the highest phenolic content and antioxidant properties, ensuring prioritization in the phytotherapeutic pipeline. *In vitro* anthelmintic assays revealed that 80% acetone extracts of *P. lentiscus*, *H. italicum picardii*, *C. mariscus*, and *L. monopetalum* were highly effective against egg hatching and larval exsheathment of the clinically relevant gastrointestinal nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis*. After, a PVPP-HPLC-ESI-MS^n approach identified potential bioactive metabolites contributing to the activity of each species. For *C. mariscus*, seasonal variations allied to organ occurrence, and despite anthelmintic effects were observed year-round, inflorescences exhibited the strongest activity against egg hatching. *In vivo* trials showed that a single oral dose of *C. mariscus* and *L. monopetalum* extracts in GIN-infected lambs had no significant effect on parasitological status but led to slight improvements in hematological parameters. In the end, this dissertation provided new insights into the biochemical potential of salt-tolerant species targeting GIN infections in ruminants, highlighting their applications in this scope and opening novel avenues for innovative anthelmintic solutions in ruminant production.

Keywords: nutraceutical; phytotherapeutical; halophytes; phenolics; nematodes; anthelmintic.

RESUMO

A procura por soluções inovadoras e sustentáveis no combate às infeções causadas por parasitas gastrointestinais em ruminantes intensificou-se nas últimas décadas, especialmente devido ao aumento exponencial e global de fenómenos de resistência parasitárias aos fármacos comercialmente disponíveis. Perante isto, de entre as várias abordagens exploradas para o controlo e gestão integrada das infeções em contexto pecuário, o uso de plantas ou de produtos derivados de plantas com propriedades bioativas contra estes parasitas, ganhou destaque devido aos múltiplos resultados promissores obtidos com diferentes espécies botânicas, ricas em compostos fenólicos, estendendo-se posteriormente a outros recursos naturais. Mais especificamente, as plantas mediterrânicas tolerantes ao sal possuem um conjunto de adaptações químicas às condições ambientais adversas e desafiantes nas quais proliferam que lhes oferecem um potencial considerável para a gestão pecuária, particularmente em áreas onde solos salinos ou degradados limitam a produtividade agrícola de espécies sensíveis à salinidade. Algumas destas adaptações conferem-lhes potencial para a aplicação como anti-helmínticos para ruminantes, embora tal tenha sido até ao momento subvalorizada. Muitas destas espécies tolerantes ao sal destacam-se por serem notáveis fontes naturais de compostos de valor acrescentado, como compostos fenólicos, vitaminas e minerais, que apresentam benefícios comprovados e sustentam os inúmeros usos tradicionais reportados, tanto na alimentação como na prevenção e tratamento de doenças humanas e condições veterinárias. Assim, o objetivo principal desta dissertação é explorar o potencial de espécies tolerantes ao sal, previamente identificadas em ambientes de influência marinha, como zonas costeiras, dunas ou sapais ao longo da região do Algarve (Portugal), com potencial para o controlo de infeções por nemátodes gastrointestinais em ruminantes, seja como plantas nutracêuticas ou como possíveis opções fitoterapêuticas. As espécies selecionadas para este estudo foram escolhidas com base em critérios como descrições etnofarmacológicas, informações fitoquímicas como o teor em compostos fenólicos, ou como possuindo atividade anti-helmíntica, e ainda acessibilidade da biomassa na sua forma selvagem. As espécies selecionadas foram assim: *Pistacia lentiscus* L., *Helichrysum italicum* subsp. *picardii* (Boiss. & Reut.) Franco, *Inula crithmoides* L., *Calystegia soldanela* (L.) R. Br., *Cladium mariscus* (L.) Pohl, *Medicago*

marina L., *Plantago coronopus* L., *Limoniastrum monopetalum* L. Boiss e *Crucianella marítima* L.. No geral, as análises de composição nutricional e química realizadas revelaram que as algumas espécies, como *H. italicum picardii*, *M. marina* e *C. soldanella* possuem potencial para aplicações nutracêuticas, essencialmente devido aos seus teores de proteína bruta ou conteúdo em macro- e micronutrientes, como potássio, magnésio, sódio, ferro, manganês e zinco. Por outro lado, *H. italicum picardii*, *P. lentiscus*, *L. monopetalum*, *C. mariscus* destacaram-se das restantes espécies estudadas como sendo ricas em compostos fenólicos, muitos deles com atividade anti-helmíntica já descrita, podendo também ser valorizadas pelas suas propriedades antioxidantes, e figurando, assim, como promissoras para o desenvolvimento de soluções fitoterapêuticas. A avaliação sazonal, dependente da espécie em questão, permitiu identificar a ou as estações do ano em que cada espécie apresentava maiores conteúdos dos compostos de interesse, antecipando maior potencial biotecnológico. Esta análise, para além da compreensão do comportamento de variação destes parâmetros com a sazonalidade, permitiu ainda priorizar as amostras de cada espécie com maior potencial para o desenvolvimento de soluções fitoterapêuticas. Ensaios *in vitro*, efetuadas com culturas laboratoriais dos parasitas em estudo, demonstraram que os extratos preparados com 80% de acetona a partir da biomassa das espécies *P. lentiscus* (colhida no Inverno), *H. italicum picardii*, *C. mariscus* e *L. monopetalum* (colhidas no Verão), foram altamente eficazes contra a eclosão de ovos e exsudação larval dos parasitas gastrointestinais clinicamente relevantes *Haemonchus contortus* e *Trichostrongylus colubriformis*. No seguimento destes estudos, foram identificados e quantificados por HPLC-ESI-MS^n vários potenciais metabolitos bioativos para cada espécie, nomeadamente flavonoides glicosilados e isómeros de ácido galoilquínico em *P. lentiscus*; ácidos cafeoilquínico e dicafeoilquínicos e formas glicosiladas de quercetina em *H. italicum picardii*; proantocianidinas, ácidos fenólicos simples e luteolina em *C. mariscus*; e flavonóides sulfatados e/ou metilados em *L. monopetalum*. O tratamento dos extratos com polivinilpolipirrolidona (PVPP) revelou que a atividade anti-helmíntica contra a eclosão dos ovos estava associada à presença de polifenóis nos extratos testados, embora estes tenham sido apenas parcialmente responsáveis pela atividade contra a exsudação larval. A análise dos resultados de composição fitoquímica, antes e depois do tratamento, em conjunto com as atividades anti-parasitárias observadas e a comparação com a literatura, permitiu a

inferência de quais compostos seriam os possíveis responsáveis pelos efeitos observados. Adicionalmente, para *C. mariscus*, foram observadas variações sazonais na atividade anti-helmíntica, particularmente associadas à ocorrência de órgãos específicos (inflorescências e folhas). Embora esta atividade se tenha verificado ao longo do ano, contribuindo para a valorização fitoquímica da espécie durante todo o ano, as amostras da biomassa coletadas no Verão e Outono foram pontualmente mais ativas, possivelmente devido á presença de inflorescências nestes períodos. De facto, as inflorescências exibiram maior atividade nos ensaios anti-parasitários *in vitro* em comparação com as folhas, especialmente contra a eclosão de ovos. Baseado nestes resultados, os extratos de duas das espécies mais ativas, *C. mariscus* e *L. monopetalum* foram selecionados para serem testados *in vivo* nos hospedeiros ruminantes relevantes. Infelizmente, estes ensaios mostraram que a aplicação de uma única dose oral de extratos em cordeiros infetados com as duas espécies de nemátodes gastrointestinais não teve efeitos significativos no estado parasitológico dos mesmos, após 18 dias de tratamento, mais especificamente na contagem de ovos excretados nas fezes. No entanto, verificaram-se ligeiros aumentos nos parâmetros hematológicos, isto é, volume globular eritrocitário, nos animais pertencentes aos grupos de tratamento com os extratos, em relação aos animais designados como grupo controlo; sugerindo a possibilidade de um potencial efeito positivo no estado geral antioxidante e imunológico dos animais. Apesar dos resultados *in vivo* terem sido maioritariamente nulos, seria importante continuar a investigar o potencial efeito destes extratos com modificações na metodologia experimental do ensaio, como dose e modo de administração dos extratos. Estes ensaios poderão ajudar na clarificação do valor anti-helmíntico dos mesmos.

Em conclusão, esta dissertação fornece novos conhecimentos sobre o potencial bioquímico de plantas tolerantes ao sal no combate a infeções por nemátodes gastrointestinais, destacando as suas aplicações fitoterapêuticas e abrindo novas perspetivas para soluções anti-helmínticas inovadoras na produção de ruminantes.

Palavras-chave: nutracêuticos; fitoterapêuticos; halófitas; compostos fenólicos; nemátodes; anti-helmínticos

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AH	Anthelmintic
AMA	Adult mortality assay
AMIA	Adult motility inhibition assay
ATCvet	Anatomical therapeutic chemical classification for veterinary medicines
Au	Autumn
C	Control group
CE	Catechin equivalents
CHO	Total carbohydrates
CI	Confidence intervals
CM	*Cladium mariscus* (treated group)
CP	Crude protein
CT	Condensed tannins
CTC	Condensed tannins content
DM	Dry matter
DMACA-HCl	4-dimethylaminocinnamaldehyde-hydrochloric acid
DMSO	Dimethyl sulfoxide
DPI	Days post-infection
DR	Dietary requirements
DW	Dry weight
EC_{50}	Half maximal effective concentration
EH	Egg hatching
EHIA	Egg hatching inhibition assay
EPG	Eggs *per* gram
EVR	Ethnoveterinary reports
FAO	Food and Agriculture Organization
F-C	Folin-Ciocalteu reagent
FEC	Fecal egg count
FECR	Fecal egg count reduction
FL	Flowers
FR	Fruits
FRAP	Ferric reduction antioxidant power
GA	Gallic acid

GAE	Gallic acid equivalents
GE	Gross energy
GIN	Gastrointestinal nematodes
HBA	Hydroxybenzoic acid
HC	*Haemonchus contortus*
HCA	Hydroxycinnamic acid
HPLC-DAD	High performance liquid chromatography-diode array
HPLC-ESI-MS^n	High performance liquid chromatography with electrospray ionization mass spectrometric detection
HPLC-Q-TOF-MS	High performance liquid chromatography quadrupole-time-of-flight mass spectrometer
HT	Hydrolysable tannins
Inf	Inflorescences
IC_{50}	Half maximal inhibitory concentration
ICNF	Instituto da Conservação da Natureza e das Florestas
IFIF	International Feed Industry Federation
Le	Leaves
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Fourth-stage larvae
LC_{50}	Half maximal lethal concentration
LC-MS	Liquid chromatography–mass spectrometry
LDA	Larval development assay
LDIA	Larval development inhibition assay
LDY	L3 larvae development yields
LE	Larvae exsheathment
LEIA	Larval exsheathment inhibition assay
LFIA	Larval feeding inhibition asssay
LM	*Limoniastrum monopetalum* (treated group)
LMIA	Larval migration inhibition assay
MP-AES	Microwave plasma-atomic emission spectrometer
MTL	Maximum tolerable levels
n.d.	Not determined
NS	Not specified

PAL	Phenylalanine ammonia lyase
PBS	Phosphate-buffered saline
PC	Proacyanidin
PCV	Packed cell volume
PD	Prodelphinidin
PEG	Polyethylene glycol
PSM	Plant secondary metabolites
PVPP	Polyvinylpolypyrrolidone
QA	ATCvet category for alimentary tract and metabolism
QD	ATCvet category for dermatological
QE	Quercetin equivalents
QG	ATCvet category for genitourinary system and sex hormones
RA	Relative abundances
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RT	Retention time
S	Stems
SEM	Standard error of mean
Sp	Spring
Su	Summer
TC	*Trichostrongylus colubriformis*
TFC	Total flavonoid content
TIPC	Total individual phenolic content
TL	Total lipids
TPC	Total phenolic content
Un.	Unspecified
USD	American dollars
UV	Ultraviolet
Wi	Winter

CHAPTER I

GENERAL INTRODUCTION AND OBJECTIVES

Parts of this introduction are published in:

Oliveira M, Hoste H, Custódio L. (2021). A systematic review on the ethnoveterinary uses of Mediterranean salt-tolerant plants: exploring its potential use as fodder, nutraceuticals or phytotherapeutics in ruminant production. *J. Ethnopharmacol. 267,* 113464. https://doi.org/10.1016/j.jep.2020.113464

1.1. PLANT BIOACTIVE METABOLITES

Initial classifications of plant organic metabolites distinguished them based on their prevalence across plants: either occurring widely or restricted to specific species, later progressing to the classical distinction into two categories, *viz.* primary and secondary metabolites (Dixon & Dickinson, 2024). Plant primary metabolites, like proteins, carbohydrates, lipids and nucleic acids, being abundant among the kingdom and biosynthesized in substantial amounts, are key metabolites in maintaining vital cellular processes for plant growth and development (Tariq et al., 2023). Other compounds, derived from the primary metabolism, are usually referred to as plant secondary metabolites (PSM) which, according to Harborne (1999), *"(...) represent substances which do not appear to have an essential role in metabolism (...) yet (...) they have a key role in protecting the plant from environmental pressures or in controlling plant growth"*.

Undoubtedly, one of the primary functions of PSM is to afford an evolutionary advantage, enhancing their ability to survive and thrive in their natural surroundings, exposed to abiotic and biotic challenges (Delgoda & Murray, 2017). It was long assumed that, in plant defense, carbon resources were diverted from the primary metabolism towards synthesis and regulation of PSM, an often-costly trade-off that prioritizes protection in detriment to growth or reproduction (Stamp, 2003). However, the scientific community has been accumulating substantial evidence of the multifunctional attributes of several PSM, not only in defense and ecological contexts but also as having functions in the primary metabolism, mitigating allocation costs, challenging humbler traditional classifications (Petersen, 2007; Neilson et al., 2013). In this context, Neilson and colleagues (2013) claimed a broader perspective on a species' PSM profile, as a complex outcome of its unique responses to environmental stressors while upholding an equilibrium with primary metabolism.

Even though PSM were once perceived as waste products, research into their biochemical properties gained momentum by the early 19th century, especially with the isolation of morphine, shifting the perspective on these metabolites towards bioactive compounds with multiple biotechnological applications (Hartmann, 2007; Jiménez-Garcia et al., 2013). In fact, Burrel (1937) stated that one of humanity's oldest pursuits has been the quest to uncover plant medicinal or toxic properties, leading to a gradual

evolution of phytochemistry – from simple plant extracts to the characterization of bioactive metabolites.

Though the term *plant bioactive metabolites* often recall PSM, several primary metabolites also have attributable pharmacological effects. However, in contrast to primary metabolites, the secondary metabolism offers greater chemodiversity, as illustrated by over 200,000 structures identified, despite a precise function remaining unknown for the majority (Hartmann, 2007). This is related to the fact that for many PSM, its biosynthesis and regulation often occurs at specialized organs, tissues or cells, and even at certain developmental stages (Wink, 2010). Nowadays, a vast number of biotechnological uses are assorted to plant-derived products and their related bioactive PSM, ranging from feed and food, pharmaceuticals and cosmetics to agricultural and ecological contexts. Yet, given the ecological role of PSM and the nearly infinite number of species and their intricate interactions, the immeasurable diversity within the PSM group remains far from being completely disclosed, and indeed, exploited (Veerporte, 1998).

According to its biosynthetic pathway, three main classes of PSM are roughly distinguished: nitrogen-containing compounds, terpenes and phenolics (Figure 1.1). In the next section, phenolic compounds will be addressed in more detail as these are the main metabolites of interest in the scope of the present dissertation.

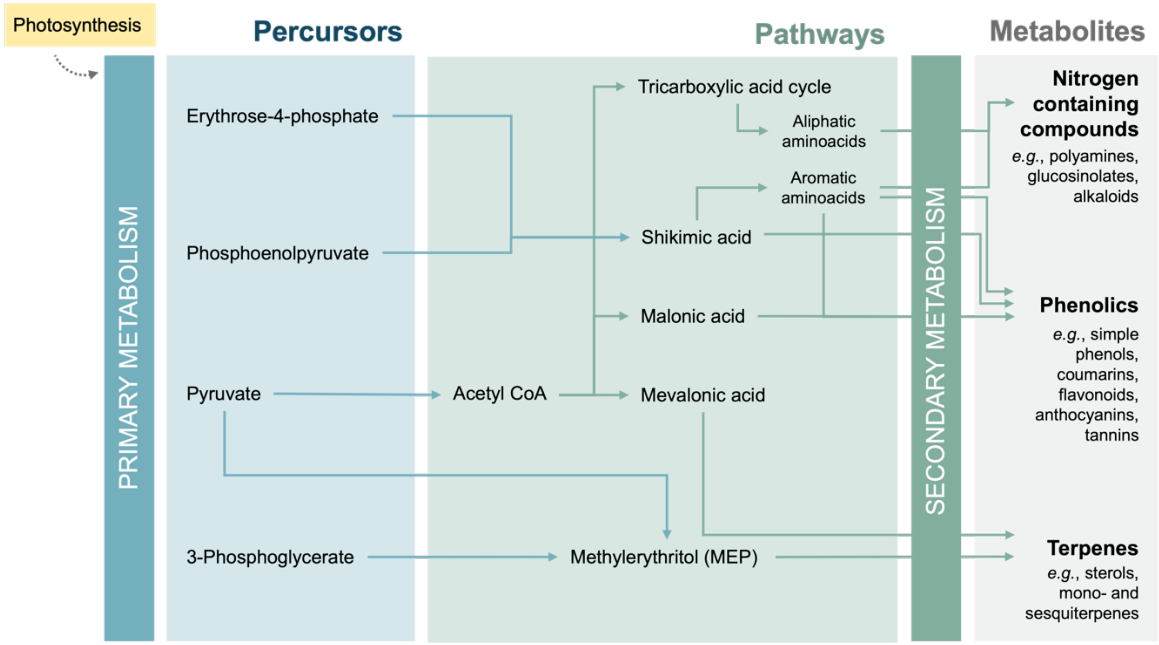

Figure 1.1 Main precursors and biosynthetic pathways of secondary metabolites (Adapted from Taiz & Zeiger, 2002; Sinha et al., 2019).

1.1.1. Phenolic metabolites

In higher plants, phenolic compounds are mostly derived from the aromatic amino acid phenylalanine via the shikimic acid pathway, yielding the precursors of an array of phenolic structures of increasing complexity (Taiz & Zeiger, 2002; Figure 1.2). The first step involves the deamination of phenylalanine, catalyzed by the key enzyme phenylalanine ammonia lyase (PAL), heading towards the formation of cinnamic acid (Taiz & Zeiger, 2002; Figure 1.2). The activity of this enzyme is usually regulated by small gene families (1-5), but can reach up to 11 or even 40-50 members (*e.g.,* cucumber or potato), their expression varying in response to ecological conditions like heat, light, salinity or fungal infections, illustrating its role in defense (Joos & Hahlbrock, 1992; Logemann, 1995; Fan et al., 2022; Amjad et al., 2024).

This diverse group foreseen by more than 10,000 structures described (Taiz & Zeiger, 2002) occurring ubiquitously and displaying a manifold of biological effects, endorses phenolic compounds as a prolific and popular PSM faction. Phenolics are generally depicted as having one or more hydroxyl substituents on, at least, an aromatic ring (Crozier et al., 2006), and are often called polyphenols; however, not all phenolics should be appraised as such. Quideau and colleagues (2011) proposed a definition for polyphenolic compounds as those *"(...) derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression"*, putting aside the monophenolic structures (*i.e.,* simple phenols) and its naturally occurring derivatives. Nonetheless, different classes are recognized based on their chemical structure, including simple phenolics, flavonoids, lignins, and condensed tannins.

Simple phenolics

Phenolic acids are the first class of metabolites derived from the deamination of phenylalanine into *trans*-cinnamic acid, following hydroxylation to *p*-coumaric acid (Figure 1.2). These commonly occur as bound soluble forms, either through esterification or glycosylation, serving also as building blocks of other phenolics of greater complexity (*e.g.*, hydrolysable tannins; Lattanzio, 2013). Phenolic acids are divided into

hydroxybenzoic (HBA) and hydroxycinnamic acid (HCA) derivatives: while HBA are molecules with a general structure C6–C1 (*e.g.,* gallic acid, vanillic acid, salicylic acid, syringic acid), HCA have a C6–C3 basic backbone (*e.g.,* cinnamic acid, caffeic acid, ferulic acid; Pietta et al., 2003; Tsao, 2010).

Flavonoids

The flavonoid branch starts out after the association of 4-coumaroyl-CoA and three molecules of malonyl-CoA leading to the formation of chalcone, a reaction catalyzed by chalcone synthase (Tsao, 2010). Afterwards, the isomerization of chalcone to flavanone occurs, yielding the precursor of many flavonoid sub-groups (Tsao, 2010; Figure 1.2). Nonetheless, coumaroyl-CoA also participates in the biosynthesis of lignins, lignans, stilbenes and coumarins (Böttger et al., 2018; Figure 1.2.).

Flavonoids are one of phenolics' biggest classes, all structures sharing a basic backbone of two aromatic rings linked by three carbon bridges (C6—C3—C6), a result of by-products derived from shikimic and malonic acid pathways (Taiz & Zeiger, 2002). Depending on the level of oxidation and pattern of modification of the aromatic ring, subclasses are defined, namely flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanidins (Böttger et al., 2018; Figure 1.2). Under natural conditions, structural variations may occur, with different attached substituents leading to, for example, prenylated (Škovranová et al., 2022), glycosylated (Isidore et al., 2024) and sulphated derivatives (Teles et al., 2018), supporting the chemical diversity of this class.

Tannins

Another group of polyphenolic compounds are tannins. In 1962, Swain & Bate-Smith portrayed tannins as "*water-soluble phenolic compounds, have molecular weights lying between 500 and 3,000, and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids and gelatin and other proteins".*

Tannins can be distinguished into two categories, namely hydrolysable tannins (HT) and condensed tannins or proanthocyannins (CT). Condensed tannins (=proanthocyanidins) originate from the flavonoid biosynthetic branch, through the combination of dihydroflavonones, flavan-3,4-diols and anthocyanidin derivatives,

having a basic backbone (C6—C3—C6)$_n$ (Lattanzio, 2013; Böttger et al., 2018). The resulting molecules are oligomeric and polymeric metabolites and can be further classified into sub-groups, such as procyanidins (*i.e.*, (epi)catechin units) and prodelphinidins (*i.e.*, (epi)gallocatechin units; Pietta et al., 2003; Crozier et al., 2006).

In contrast, HT derive from the shikimate pathway, entailing a central sugar molecule and hydroxyl groups esterified by gallic acid (gallotannins) or gallic acid and hexahydroxydiphenic acid (ellagitannins) and hydrolysis leads to the release of their basic units, inspiring their name (Chung et al., 1998; Crozier et al., 2006; Lattanzio, 2013).

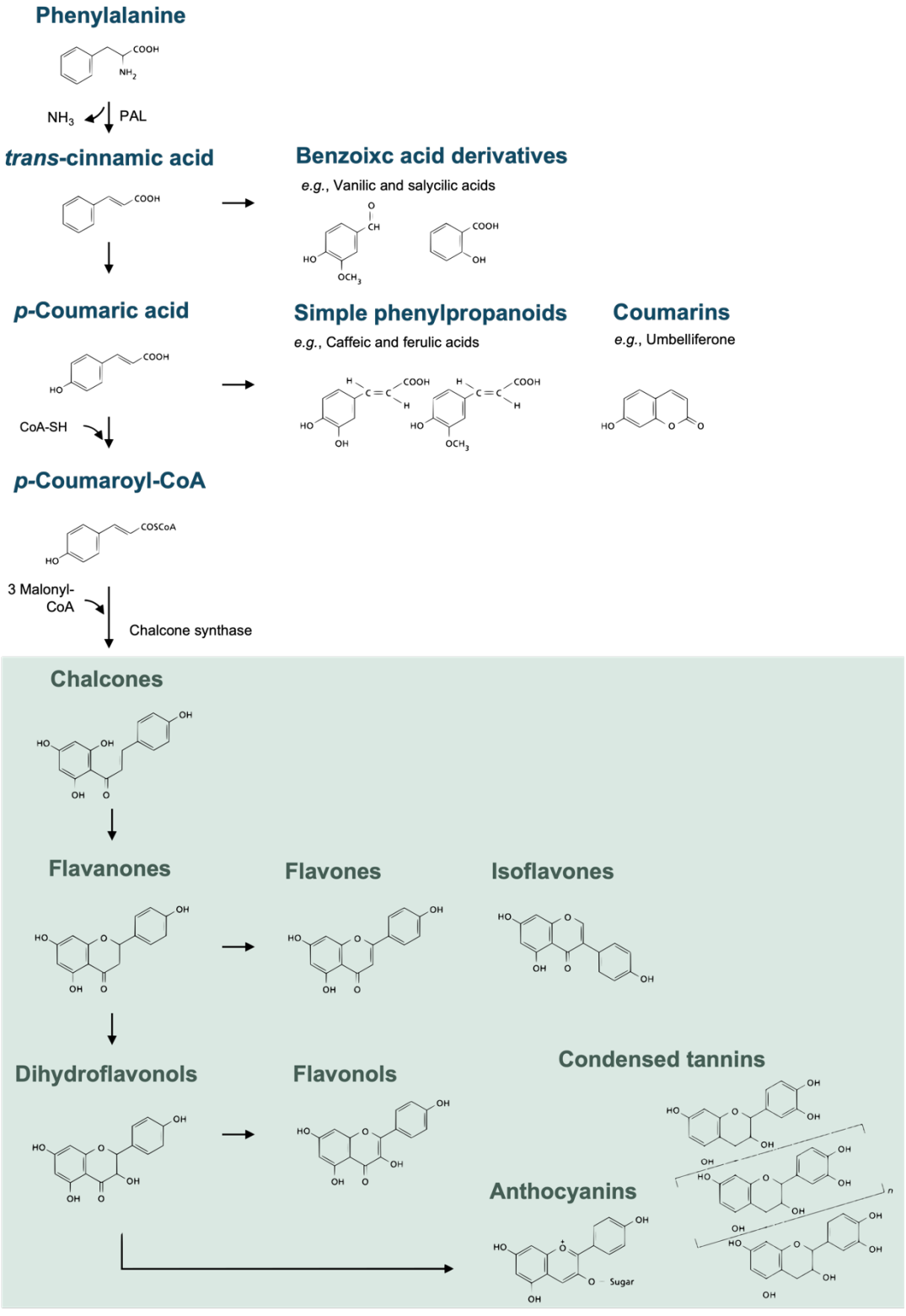

Figure 1.2 Phenolic compound classes derived from phenylalanine, including the flavonoid branch, highlighted in green (Adapted from Taiz & Zeiger, 2002).

1.1.2. Factors affecting phenolics' biosynthesis

As stated earlier in section 1.1., the biosynthesis of PSMs is recognized as a key component of a plant's defense system, such as in offering structural support, protecting plants against predators and pathogens, adapting to environmental conditions or functioning as allelochemicals (Taiz & Zeiger, 2002).

Among these, environmental parameters have been broadly associated with both qualitative and quantitative changes in plants phenolic production. As a few examples, in controlled trials, excessive light stress, ultraviolet (UV) irradiance, and sodium chloride stress were found to enhance the biosynthesis of antioxidant flavonoids in *Ligustrum vulgare* L. 1753 plants, particularly quercetin and luteolin glycosides (Tatini et al., 2004; Agati et al., 2011). Boughalleb & Denden (2011) reported that under increasing salinity levels (0-800 mM NaCl), the overall phenolic content of *Nitraria retusa* Asch. 1876 increased, whilst *Atriplex halimus* L. 1753 leaves accumulation peaked at 100 mM. In the presence of cadmium (Cd; 5 μg Cd g^{-1} soil), the flowering plant *Erica andevalensis* Cabezudo & J. Rivera, 1980, exhibited an increased concentration of phenolic compounds in relation to control plants, particularly cinnamic acid derivatives, epicatechin, and rutin, whilst in higher amounts the phenolic content decreased (Márquez-García et al., 2011). *Fagopyrum esculentum* Moench 1794, sprouts treated with increasing levels of NaCl showed higher phenolic contents and antioxidant effects, relying on the contents of the individual metabolites isoorientin, orientin, rutin, and vitexin (Lim et al., 2012). Similarly, *Olea europaea* L. 1753, leaves under water stress exhibited higher phenolic content than controls, with increased accumulation of oleuropein, verbascoside, luteolin 7-*O*-glucoside, and apigenin 7-*O*-glucoside (Mechri et al., 2020).

The role of these abiotic triggers is particularly relevant in the Mediterranean context. Sunlight and UV exposure, temperature, salinity and nutrient-deficient soils are main environmental stressors that characterize the region, promoting phenolics biosynthesis in Mediterranean plants, with intra- and interspecific variations (Di Ferdinando et al., 2014). Aligned with this, seasonal and geographical fluctuations of phenolics and their related biological effects have been reported for Mediterranean plant species and associated with environmental parameters as well as habitat types (Ksouri et al., 2008; Gil et al., 2014; Bautista et al., 2016; Gori et al., 2020; Pereira et al., 2024).

Interestingly, Bautista and colleagues (2016) observed that while there were strong positive relationships between phenolic contents with altitude and water stress, the correlation with salinity was weak. The authors noted that, in general, highly salt-tolerant species growing in salt marshes had lower amounts of antioxidant phenolics, hypothesizing that, as these are well-adapted to their natural habitats, salinity may not be a trigger (Bautista et al., 2016), which was also supported by past studies (Gil et al., 2014).

However, aside from abiotic factors, plant-related factors, like species, phenological stage, and organs, are also key contributors to variations in phenolic patterns. For example, Lopes and colleagues (2016) described a wide range of total phenolic (38.8-278 mg GAE g^{-1} DW), flavonoid (0.43-10.4 mg GAE g^{-1} DW) and tannin contents (6.63-43.4 mg catechin eq. g^{-1} DW) for Mediterranean salt-tolerant plant species, several harvested at the same site and time. In another work, Di Ferdinando and colleagues (2014) advocated that variations in the phenolic composition and concentration among Mediterranean co-occurring species support the multifunctional roles of these metabolites in plant species-specific ecological responses. For the salt-tolerant *Crithmum maritimum* L. 1753, and *Limonium densiflorum* (Guss.) Kuntze, the highest levels of phenolic compounds and flavonoids were recorded in the flowering stage (Medini et al., 2011; Jallali et al., 2012). Indeed, significant variations among anatomical organs have been recorded for several salt-tolerant species, including *Tamarix gallica* L. 1753, *Kali turgidum* (Dumort.) Gutermann, 2011 (syn. *Salsola kali* L.*), Limoniastrum monopetalum (L.)* Boiss. 1848 and *Limonium algarvense* Erben 1978, either in amount and/or class of metabolites (Ksouri et al., 2008; Trabelsi et al., 2012; Rodrigues et al., 2015). In addition to plant-related traits, biotic triggers such as pathogen infections can also affect phenolics composition, as observed, for example, for the infected red raspberry (*Rubus idaeus* L. 1753) with the fungi species *Xenodidymella applanata* (Niessl) Q. Chen & L. Cai 2015 (syn. *Didymella applanata*) and *Leptosphaeria coniothyrium* (Fuckel) Sacc. 1875, which had increased levels of flavan-3-ols and tannins in comparison to healthy controls (Mikulic-Petkoysek et al., 2014).

While it is relevant to consider potential fluctuations in bioactive plant phenolics, studying abiotic and biotic triggers and plant-related factors can be challenging, as they often interact simultaneously rather than independently in natural environments – thereof,

the plant's ecological frame is essential for determining the extent of metabolite's production.

1.1.3. Biological activities

The antioxidant, radical scavenging and metal chelation features of phenolic metabolites are the core of their therapeutic effects, along with their modulatory functions at gene- and cellular levels (Soobrattee et al., 2005). Their strong antioxidant and radical scavenging properties are centered on the aptitude of the phenolics' hydroxyl groups to donate a hydrogen atom or an electron to a free radical, coupled with the capacity of the aromatic ring to stabilize an unpaired electron through delocalization (Pietta et al., 2003). Indeed, it is virtually impossible to address phenolics' bioactivities without recalling their antioxidant and radical scavenging abilities – these are among their most studied biological traits, and correlations between these parameters are frequently reported (Soobratte et al., 2005; Maisuthisakul et al., 2008; Piluzza & Bullita, 2011; Stanković et al., 2023) as well as the individual activity of phenolic metabolites (Rice-Evans et al., 1996;1997).

Consistent with the previous section 1.1.2., the antioxidant capacity of phenolics evokes their protective role in plants. When exposed to abiotic and biotic stressors, plants can overproduce reactive oxygen species (ROS), triggering their antioxidant defense arsenal – including enzymatic and non-enzymatic metabolites – aiming at safeguarding key cellular components (*e.g.*, proteins, lipids, DNA; Hasanuzzaman et al., 2012). Pairwise, antioxidant and radical-scavenging properties are comparably useful in the management of oxidative stress-related human and animal diseases. Indeed, extensive research has been conducted and compiled in different review works, examining the role of plant-based phenolics in mitigating oxidative stress and inflammatory-related disorders, such as in promoting cardiovascular health (Lutz et al., 2019), supporting neuroprotection (Tavan et al., 2024), aiding in the management of metabolic disorders like obesity (Rodríguez-Pérez et al., 2017), diabetes (Deka et al., 2022) and cancer (Wahle et al., 2010) as well as to ameliorate skin-aging associated disorders (Csekes & Račková, 2021). Additional bioactivities, reflecting their role in plant defense against predators, include antimicrobial properties such as antibacterial (Lobiuc et al., 2023), antiparasitic activities (Soto-Sánchez, 2022; Hoste et al., 2015) and antiviral effects (Montenegro-

Landívar et al., 2021), against pathogens affecting humans and animals. Aligned with this, Mediterranean salt-tolerant plants exhibit various of these pharmacological effects, supporting their medicinal claims, and often associated with their unique phenolic profiles (Ksouri et al., 2012a,b; Lopes et al., 2016; 2023; Stanković et al., 2023).

In the end, a wide collection of bioactivities is ascribed to phenolic molecules; however, it is important to consider that their bioactive effects frequently depend on the structural configuration of the metabolite(s) in question (Rice-Evans et al., 1996; 1997), together with the respective concentration within plant matrices (Jacobo-Velázquez & Cisneros-Zevallos, 2009). For instance, phenolic compounds from different classes possess varying radical scavenging capacities, largely influenced, for example, by the number of hydroxyl groups or glycosylation patterns (Rice-Evans et al., 1997; Soobrattee et al., 2005). Furthermore, in complex plant matrices, synergistic, additive or antagonistic effects can occur (Peyrat-Maillard et al., 2003; Cirico & Omaye, 2006), resulting in bioactive properties that are a combination of multiple interactions.

1.2. PLANT BIOACTIVE PRODUCTS FOR RUMINANT PRODUCTION

Ruminant production is a significant agricultural and economic sector in the Mediterranean region, with approximately 267 million cattle, sheep, and goats, according to the statistical database of the Food and Agriculture Organization (FAO, 2019). In 2023, the global animal health market was estimated to be worth 62.40 billion USD, with production animals (ruminants, poultry, swine, and fish) dominating the market (Grand View Research, 2024). In addition, the nutraceutical industry generates over 250 billion USD annually, with animal health applications being particularly attractive, primarily due to affordability and often safety profiles (Gupta et al., 2019). Still, the development of novel veterinary products is often based on research conducted for human applications, with limited attention given to the identification and testing of bioactive products specifically targeting ruminants (Rochfort et al., 2008).

A renewed interest in the reduction of chemically synthesized substances in ruminant production has been boosted by the banning of antibiotics as growth promoters in animal feed (European Parliament and Council Regulation (EC) No. 1831/2003), regulations on organic production (Council Regulation (EC) No/834/2007), consumers consciousness towards animal welfare (European Commission, 2015) and food safety-

related issues (European Commission, 2019). Altogether, these set a shift in the perception of using plants or their bioactive products as effective and sustainable alternatives for improved animal nutrition, health, welfare, productivity and meat and milk quality.

Under the ruminant context, predominantly four key formulations are pursued for exploitation, either of synthetic – drugs and feed additives – or of natural origin – nutraceuticals and phytotherapeutics, though some definitions lack consensus (Hoste et al., 2015; Torres-Fajardo et al., 2020). That is the case of plant nutraceuticals, whose descriptions vary the most, but ultimately, represent the intersection of food/feed, nutrients and a drug (Gupta et al., 2019; Torres-Fajardo et al., 2020), which aligns with the proposed definition by Frank and colleagues (2019) for foods, that a nutraceutical is ‘’*a compound or mixture of compounds present in food or food supplements intended to exert a therapeutic effect*’’.

The concepts of nutraceuticals and phytotherapeutics given by Hoste and colleagues (2015) head towards their use against gastrointestinal nematodes (GIN), and thereof, were followed herein. In agreement to the previous definitions, the model of nutraceutical plants recalls to plants and plant-based products that can be used as feed (or part of a feed) for long-term periods, and which combine nutrition and anthelmintic effects on animals (Hoste et al., 2015). It relies on the premise that plant bioactive metabolites are effective concentrations at dietary levels and that the animals voluntarily eat the plant and/or plant products for a sufficient length of time (Hoste et al., 2015). Plant bioactive metabolites that endow forages with nutraceutical features are also the core of herbal remedies (phytotherapeutics) used in traditional veterinary practices. In contrast to nutraceuticals, the use of phytotherapeutics implies the administration of the active formulation to animals for a short-term period, aiming for the treatment of a specific disease (Hoste et al., 2015). These formulations include plant-based products, such as extracts, fractions and/or essential oils, *i.e.,* enriched with bioactive metabolites, for a particular therapeutical use.

Within both contexts, polyphenols and polyphenol-rich plants have been in the spotlight, reliant on their diverse range of bioactive properties, previously discussed in section 1.1.3. Likewise, numerous works claim their use in ruminant production beyond GIN infections, such as in improving ruminant nutrition through modulation of ruminal

fermentation (Rira et al., 2015; De Paula et al., 2016), improving the overall antioxidant status or managing oxidative stress-related events, like reproduction and fertility (Macías-Cruz et al., 2018; Ciampi et al., 2020; Bešlo et al., 2022), the fatty acids content of meat and milk, through modulation of biohydrogenation (Guerreiro et al., 2021; Frutos et al., 2020), as well as tackling environmental concerns such as methane emissions (Ma et al., 2017).

Given this thesis primary focus on delivering novel anthelmintic plant-based products, the following section will delve more deeply into gastrointestinal nematodes biology, their impact on ruminant animals and current control approaches.

1.3. GASTROINTESTINAL NEMATODES (GIN)

1.3.1. Life cycle and parasite biology

Gastrointestinal parasites (Phylum Nematoda, Order Rhabditida) are a range of helminth parasites that infect the digestive system of ruminant animals, resulting in a veterinary disorder known as parasitic gastroenteritis (Charlier et al., 2018; Zajac & Garza, 2020). The main relevant GIN species in Europe, belonging to the family Trichostrongylidae, and its respective ruminant hosts, are represented in Table 1.1.

Table 1.1 Main relevant species of gastrointestinal nematodes (Trichostrongylidae) infecting ruminant animals and the location of adult worms in the host (Adapted from Charlier et al. 2018).

Species	Host	Location
Haemonchus contortus (Rudolphi, 1803)	Cattle, sheep, goats	Abomasum
Teladorsagia circumcincta (Stadelmann, 1894)	Sheep, goats	
Ostertagia ostertagi (Stiles, 1892) Ransom, 1907	Cattle	
Trichostrongylus axei (Cobbold, 1879) Railliet & Henry, 1909	Cattle, sheep, goats	
Trichostrongylus colubriformis (Giles, 1892) Ransom, 1911	Cattle, sheep, goats	Small intestine
Cooperia oncophora (Railliet, 1898) Ransom, 1907	Cattle	
Nematodirus spp. Ransom, 1907	Cattle, sheep, goats	

These parasites have a direct life cycle, *i.e.*, only one host is required, which consists of 1) a free-living stage that develops in the environment and 2) a parasitic stage

that develops inside the host (Figure 1.3). The biological cycle is comparable for most species: the beginning of the free-living stages is dictated by the excretion of the eggs, produced by female worms, by infected animals to the environment. Then, first-stage larvae (L1) develop inside the eggs until hatching, and once in feces, L1 molts into second (L2) and third-stage (L3) larvae.

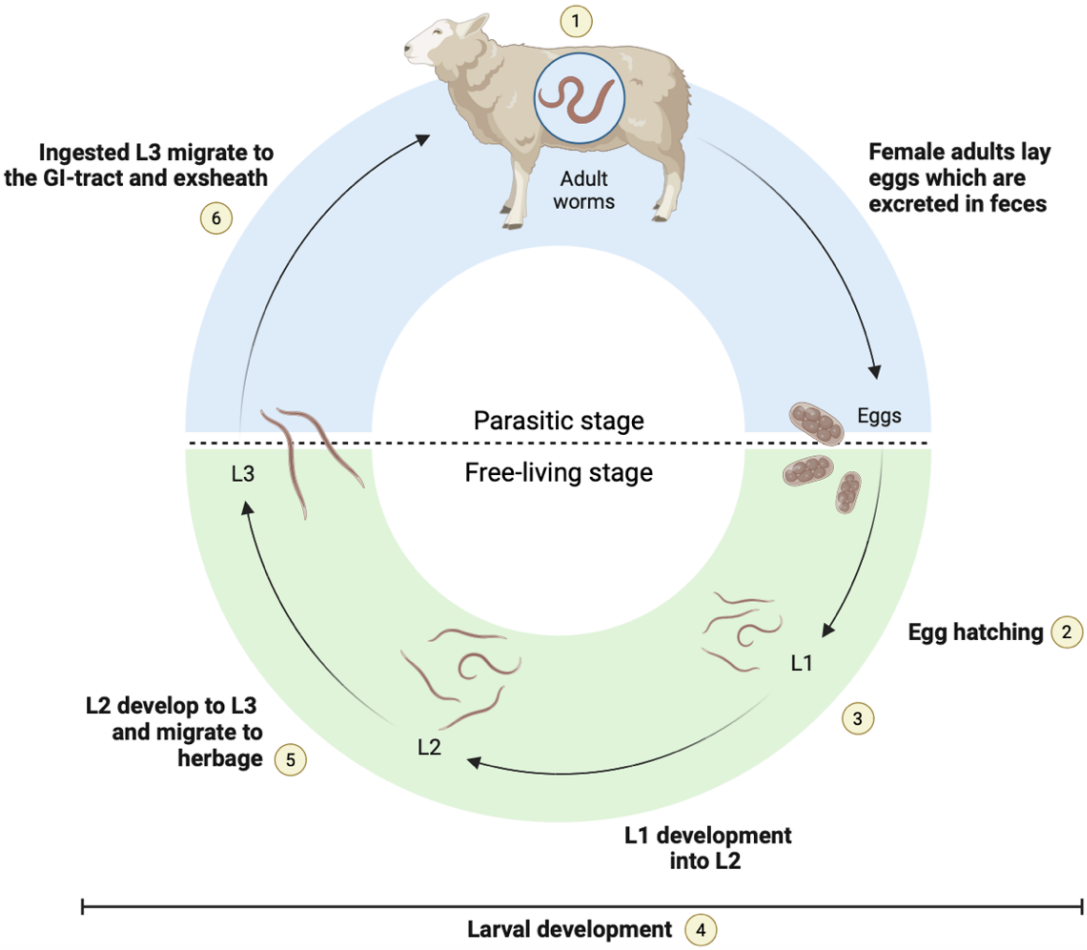

Figure 1.3 Schematic overview of the biological life cycle of gastrointestinal nematodes involving a free-living stage in the pasture and a parasitic stage inside the host; numbers represent *in vitro* assays frequently used to assess the anthelmintic efficacy of novel drugs or plant-based products against different parasite life stages (Created using BioRender.com; Adapted from Jackson & Hoste, 2010; Hoste et al., 2012;2015). **1)** Adult Motility Inhibition Assay (AMIA); **2**) Egg Hatching Inhibition Assay (EHIA); **3**, Larval Feeding Inhibition Asssay (LFIA); **4**, Larval Development Inhibition Assay (LDIA); **5**, Larval Migration Inhibition Assay (LMIA) and **6**, Larval Exsheathment Inhibition Assay (LEIA).

L1 and L2 stages that feed on bacteria and other microorganisms in feces are more susceptible to unfavorable conditions than L3 larvae, mainly because of the retained cuticular sheath from the L2 stage, providing extra protection against external stressors (Roeber et al., 2013; Zajac & Garza, 2020; Figure 1.4). On the other hand, the protective

double L3 sheath also blocks feeding, and thus, depending on species, L3 are more susceptible under extreme drought or cold periods, when energy reserves drop (O'Connor et al., 2006). Regarding morphological traits, the length of the L3 sheath tail extension is among the main discriminatory features for species identification: for example, while *T. colubriformis* spp. L3 larvae sheath tail is around 33 µm extension and has no filament in *H. contortus*, it is about 2-2.7-fold longer (van Wyk & Mayhew, 2013; Figure 1.4).

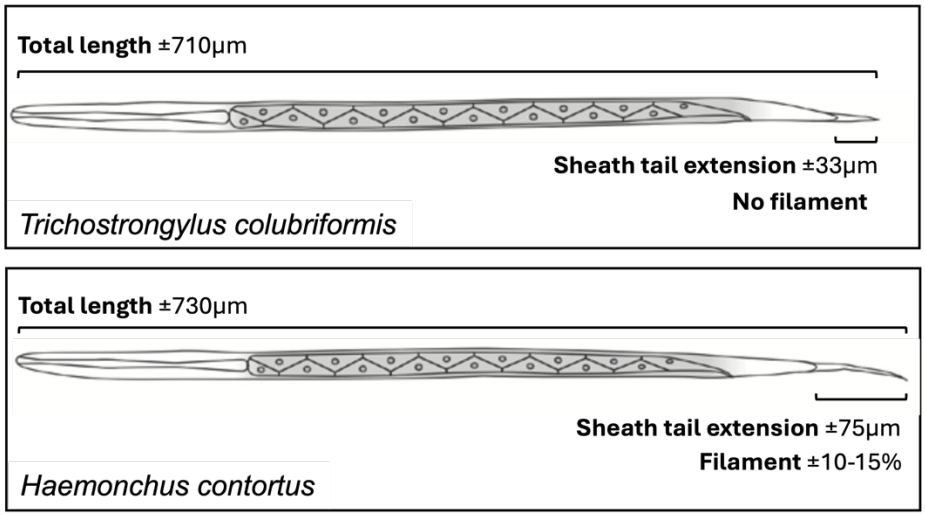

Figure 1.4 Illustration of morphological differences between third-stage larvae of *T. colubriformis* and *H. contortus* (Adapted from van Wyk & Mayhew, 2013).

In tropical and subtropical regions, the average lifespan of L3 is 1-3 months, whereas in temperate conditions, it can be extended from 6 up to 18 months, depending on the GIN species (Torres-Acosta & Hoste, 2008). Under optimal conditions, L3 larvae migrate horizontally to the herbage and vertically to the top, where they will be ingested during grazing. Upon ingestion, the drastic change in environment leads to a process called exsheathment of L3 larvae (Figure 1.5), an evolutionary strategy of parasitic nematodes where the outer cuticle (sheath) is left behind, setting the beginning of the parasitic stage (Rogers & Sommerville, 1957). This process occurs rapidly in the part of the gastrointestinal tract prior to the location where the adult parasite will establish: abomasal species will exsheath in the rumen and those established in the small intestine will exsheath in the abomasum (Hertzberg et al., 2002). Investigations revelead that, in the case of *H. contortus*, 90% of L3 larvae were exsheathed in 1h after incubation in the rumen fluid, while *T. colubriformis* reach its peak 2 hours upon transferred to the abomasum (71%; Hertzberg et al., 2002). Recently, it has been found that the main

biological stimuli triggering this exsheathment process involve heat shock and high CO_2 levels, acting in a synergistic manner (Bekelaar et al., 2018). Exsheathed L3 larvae migrate to the abomasum or intestinal mucosa, where they will develop to L4 and, lastly, to the adult stage. During the L4 stage, nematodes can undergo a temporary developmental arrest, a phenomenon known as "hypobiosis", which is an adaptative strategy of the parasite to ensure transmission under future favorable ecological conditions (Gibbs, 1986). The cycle restarts when females produce eggs that will further be excreted by the host through feces, usually 3-4 weeks after the exsheathment process takes place (Roeber et al., 2013).

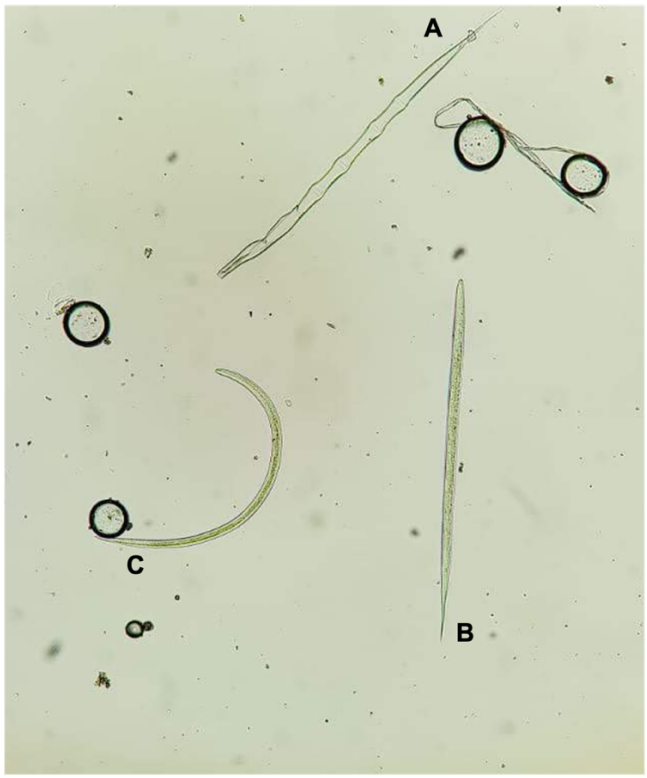

Figure 1.5 Microscopic visualization of *H. contortus* **A**) L3 ensheathed larvae, **B**) empty sheath left behind and **C**) L3 exsheathed larvae (40x magnification; photo by the author).

1.3.2. Epidemiology and clinical outcomes

Interactions within the environment-parasite-host triad shape the dynamics of GIN infections in outdoor settings, playing a key role in developing effective control strategies as well as on enhancing animal welfare and productivity (Emery et al., 2016).

First, amongst biological variations between GIN species, female fecundity is a crucial factor that ensure parasite's success: in naturally contaminated pastures, *H. contortus* female adults display a daily egg output range of more than 5,000 eggs per

female, whilst *Trichostrongylus* spp. females may excrete a few hundred and *Nematodirus* spp. a dozen of eggs per female a day (Coyne et al., 1991). Nonetheless, the parasite-environment relationship deeply influences disease transmission, since abiotic conditions are long recognized as key determinants in the development and survival of free-living stages, such as temperature, rainfall and soil moisture, reliant on each species 'ecological needs (O'Connor et al., 2006; Stromberg et al., 1997; Khadijah et al., 2013). As an example, while *T. colubriformis* has marginal egg hatching at 10 ºC, *T. axei* eggs successfully hatch at this temperature (reviewed by O'Connor et al., 2006). Regarding L3 infective larvae, *H. contortus* and *T. colubriformis* succeed in warm and moisty conditions, whereas *T. circumcincta* is best adapted to cool, moisty and sub-freezing winters (O'Connor et al., 2006). Furthermore, humidity, rainfall and temperature are also critical for a successful migration of L3 from feces to herbage, or else in dry conditions, larval accumulate in feces or in the base of the vegetation, lowering their chances of being ingested during grazing (Silva et al., 2008; Santos et al., 2012; Wang et al., 2014). More recently, epidemiological tools were developed considering these variables, aiming at predicting the seasonal dynamics of GIN infections while offering support in the elaboration of control approaches, especially under the current climate change context (Rose Vineer et al., 2015; 2020).

Once the L3 larvae reach the top of the herbage, host-specific factors may limit ingestion and subsequent infection. For instance, the predominantly browsing behavior of goats in comparison to the grazing behavior of sheep has been suggested as a regulation strategy to counteract the parasitic infection: while goats avoid ingestion of L3, sheep developed higher immune efficacy against these parasites (Hoste et al., 2001; 2010). Other host-related factors that will be determinants for infection include nutrition, age, gender, immunity, and genetics (Torres-Acosta & Hoste, 2008; Hoste et al., 2016; Hendawy, 2018). For instance, young animals and adults with a compromised immune system are usually more susceptible to parasite infections than healthy adults, mainly due to the lack of an effective immune response (Zajac, 2006; Hendawy, 2018).

In general, GIN infections are of chronic and subclinical nature, mostly impacting productivity, but severe clinical outcomes with high mortality may occur in cases of mass infections, particularly with *H. contortus* (Charlier et al., 2018). Clinical signs range from decreased appetite and weight gain to diarrhea, weight loss, anemia and bottle jaw in

heavier infections (Zajac, 2006). Nevertheless, pathogenicity depends on the parasite species: *H. contortus* is a hematophagous parasite, causing predominantly severe to fatal anemia, while *Trichostrongylus*, *Ostertagia*, *Teladorsagia* spp. infected animals usually present signs like diarrhea and weight loss (Zajac, 2006; Charlier et al., 2018). In the end, GIN infections significantly impact animal productivity, either by reducing feed intake, draining energy supplies for immune response, or through direct tissue damage (Charlier et al., 2018), but often lead to reduced yields of animal-derived products (*e.g.,* milk, wool; Arsenopoulos et al., 2021).

1.3.3. Synthetic anthelmintic drugs

For the past 65 years, the chemical arsenal used to fight GIN infections in ruminants derived from four families of anthelmintic drugs, namely, benzimidazoles, imidazothiazoles, macrocyclic lactones, and amino-acetonitrile derivatives (Table 1.2; Kotze & Prichard, 2016). However, its indiscriminate use has led to exponential reports of anthelmintic resistance and multi-drug resistance in all continents (Papadopoulos et al., 2012; Veríssimo et al., 2012; Falzon et al., 2013; Tsotetsi et al., 2013; Playford et al., 2014; Chandra et al., 2015), exacerbating concerns on how to control these nematodes effectively. Additionally, growing worries about drug residues in the environment and food chain, along with the potential for disturbances on the development of natural immunity, have led to increasing restrictions on their use (Charlier et al., 2018).

Table 1.2 Classes of anthelmintic drugs currently in use and its main targets in parasites (adapted from Kotze and Prichard, 2016; Arsenopoulos et al. 2021).

Drug Class	Drug	Release Date	Parasite target
Benzimidazoles	Thiabendazole	1961	ß-tubulin
	Parbendazole	1966	
	Fenbendazole	1971	
	Albendazole	1979	
	Oxfendazole	1975	
	Mebendazole	1971	
Salicylanilides	Closantel	1982	-
Organophosphates	Naphthalophos	1960s	-
Imidazothiazoles	Levamisole	1965	Nicotinic acetylcholine receptor genes
Macrocyclic lactones	Ivermectin	1981	Glutamate- gated chloride ion channels P-glycoprotein gene
	Abamectin	1985	
	Moxidectin	1992	
	Doramectin	1993	
	Eprinomectin	1996	
Amino-acetonitrile derivatives	Monepantel	2009 (New Zeland) 2010 (South America and Australia) 2011 (Europe)	Nicotinic acetylcholine receptor genes

1.3.4. Anthelmintic value of plant-derived products

In 2006, Krecek & Waller stated that "*the exclusive reliance on anthelmintic drugs to control internal helminths (GINs) of livestock sounds inappropriate and ultimately unsustainable".* This concern led to the proposal of integrated approaches aiming at more sustainable control of parasitic nematodes, highlighted in different reviews (Molento, 2009; Jackson et al., 2009; Hoste & Torres-Acosta, 2011; Charlier et al., 2018; 2024). The authors sustain this "integrational" concept on the use of a combination of complementary control solutions, targeting the disruption of the parasite's life cycle: 1) reduction of interactions between host and the infective L3 stage, 2) stimulation of the host response, and 3) modulation of the worm biology (Hoste & Torres-Acosta, 2011; Charlier et al., 2024). These can aid in reducing reliance on anthelmintics, by a "*basket*

of options", comprising the use of grazing management strategies (*e.g.*, rotational grazing), nematode-destroying fungi, selective breeding, improved nutrition, vaccines and plant-based options (Krecek & Waller, 2006; Charlier et al., 2024).

Included in the last solution are non-conventional anthelmintic treatments, such as plants and their bioactive products. The number of plant materials tested for their anthelmintic properties peaked in the last decades, with particular emphasis on polyphenol-rich plants (Hoste et al., 2012; 2015). This is mostly accounted to the discovery of the anthelmintic effects of nutraceutical legume forages, including bird foot trefoil (*Lotus corniculatus* L. 1753), big trefoil (*L. pedunculatus* Cav. 1793), sulla *(Hedysarum coronarium* L. 1753; Niezen et al., 1995; 1998a, b; 2002) and sainfoin (*Onobrychis viciifolia* Scop. 1772; Paolini et al., 2003; 2004), which was linked to the high polyphenolic content of these species. These works continue to inspire further investigations on the anthelmintic effects of other natural resources, such as algae (Taki et al., 2020) and agro-industrial by-products (Hoste et al., 2022).

In 2012, Sandoval-Castro and colleagues summarized plant extracts tested *in vitro* and *in vivo* against GIN in different livestock species. At that time, most plant extracts were evaluated against GIN of poultry, deer and cattle, and few investigated fractions enriched in certain metabolite classes, like tannins, saponins or sesquiterpene lactones (Sandoval-Castro et al., 2012). Later, in 2017, Spiegler and colleagues comprehensively reviewed plant extracts and/or fractions rich in polyphenols (tannins, flavonoids) tested against different GIN species and life stages from 2001 up to the end of 2016. Of 63 species belonging to 21 families, only 6 salt-tolerant species were investigated for their anthelmintic effects *in vitro* (Table 1.3; Spiegler et al., 2017). Despite polyphenols, other classes of plant metabolites have also been described of anthelmintic interest, like terpenes and saponins (Sandoval-Castro et al., 2012; Santos et al., 2019; Liu et al., 2020). Still, from 34 compounds derived from plants evaluated either *in vitro* or *in vivo* for their anthelmintic properties, phenolic compounds represented the majority (38.2%), followed by terpenoids (17.6%), alkaloids (14.7%), saponins (5.9%) and lipids (2.9%) Table 1.4; Liu et al., 2020). Chlorogenic acid, caffeoyl and coumaroyl derivatives, isokaempferide, and gallic acid shown *in vitro* promising results on egg hatching of *H. contortus*, while rutin, nicotiflorin, narcissin and epicatechin were active on the larval migration process of this parasite (Table 1.4). Nevertheless, the *in vivo* effects were only ascertained for

luteolin, epicatechin and oxytyroside, using mice, goats and calves as models, respectively (Table 1.4). More recently, Sprengel Lima and colleagues (2021) tested the activity of several phenolic compounds belonging to different classes isolated from *Pterogyne nitens* Tul., 1843 in the larval development and egg hatching of *H. contortus*. Phenolic acids were the most active on egg hatching inhibition assay (EHIA; EC_{50} caffeic acid = 1.48 μg mL^{-1}; EC_{50} ferulic acid = 0.56 μg mL^{-1}; EC_{50} gallic acid = 4.93 μg mL^{-1}), while the flavones sorbifolin and pedalitin and the flavan-3-ol ourateacatechin were considered inactive (EC_{50} > 3000 μg mL^{-1}). In larvae development assay (LDA), the phenolic acids remained quite active (EC_{50} ranging from 22-33 μg mL^{-1}) followed by the flavone pedalitin (EC_{50} = 83 μg mL^{-1}), quercetin (EC_{50} = 231 μg mL^{-1}), the flavonol rutin (EC_{50} = 104 μg mL^{-1}), and the flavan-3-ol ourateacatechin (EC_{50} = 989 μg mL^{-1}). In 2022, Olmedo-Juárez and colleagues (2022) identified kaempferol, quercetin, coumaric acid, ferulic acid, luteolin 7-*O*-rhamnoside, quercetin 3-*O*-rhamnoside, and a caffeoyl derivate as major compounds detected in the most ovicidal fractions of medicinal *Pithecellobium dulce* (Robx.) Benth against *H. contortus*. The authors highlighted the synergistic and antagonistic effects occurring in the subfractions between the phenolic acids and flavonoids (Olmedo-Juárez et al., 2022), supported by other prior works (Mancilla-Montelongo et al., 2019).

Table 1.3 Salt-tolerant species polyphenol-rich extracts and/or fractions evaluated for its *in vitro* anthelmintic properties (Adapted from Spiegler et al. 2017). EHIA, Egg hatching inhibition assay; LEIA, Larval exsheathment inhibition assay; LMIA, Larval migration inhibition assay. Un. unspecified. LC_{50}, concentration that is lethal to 50% of the individuals.

Family/Species	**Plant part**	**Extract**	**Parasite species**	***In vitro* assay**	**Effective concentration** ($\mu g\ mL^{-1}$)	**Ref.**
Acanthaceae						
Avicennia germinans (L.) L., 1764	Leaves	70% acetone:water	*Haemonchus contortus*	EHIA	2400	[1]
Anacardiaceae						
Pistacia lentiscus L. 1753	Leaves	70% ethanol:water ethanol water	*Teladorsagia circumcincta* *Trichostrongylus colubriformis*	LEIA	1200 2.4 (70% aqueous ethanolic)	[2]
	Leaves	70% acetone:water	*Haemonchus contortus*	LMIA	n.d.	[3]
Combretaceae						
Laguncularia racemosa (L.) C.F. Gaertn.1807	Leaves	70% acetone:water	*Haemonchus contortus*	EHIA	2400	[1]
Fabaceae						
Hedysarum carnosum Desf. 1799	Un.	70% acetone:water	*Haemonchus contortus*	LEIA	≥150	[4]
Robinia pseudoacacia L. 1753	Leaves	70% acetone:water	*Caernobithis elegans*	Lethality	LC_{50}=0.73 mg mL^{-1}	[5]
Rhizophoraceae						
Rhizophora mangle L. 1753	Leaves	70% acetone:water	*Haemonchus contortus*	EHIA	1200	[1]

[1] Vargas-Magaña et al. 2014; [2] Azaizeh et al. 2013; [3] Manolaraki et al. 2010; [4] Aissa et al., 2016; [5] Katiki et al., 2013.

Table 1.4 Phenolic compounds isolated from plant species with anthelmintic properties against gastrointestinal nematodes infecting ruminants (Adapted from Liu et al. 2020). IC50, concentration results in a 50% inhibition; n.d. not discriminated. LMIA, Larval Migration Inhibition Assay; AMA, Adult Mortality Assay; EHIA, Egg Hatching Inhibition Assay; LDA, Larval Development Assay; LEIA, Larval Exsheathment Assay.

Compound	**Plant species**	**Parasite species**	***In vitro* assay**	***In vitro* results**	***In vivo* model**	**Ref.**
Rutin	*Persea americana* Mill., 1768	*Haemonchus contortus*	LMIA	IC_{50} = 30 μg mL^{-1}	Goat	[1]
	Onobrychis viciifolia Scop. 1772	*Haemonchus contortus*	LMIA for 3h	Migration reduced by 25% at 1965	n.d.	[2]
Nicotiflorin	*Onobrychis viciifolia*	*Haemonchus contortus*	LMIA for 3h	Migration reduced by 30% at 2018	n.d.	[2]
Narcissin				Migration reduced by 30% at 1921	n.d.	
Luteolin	*Ajania nubigena* (Wall. ex DC.) C.Shih, 1979	*Trichuris muris*	AMA after 12 h *in vitro*/ worm burden *in vivo*	IC_{50} = 9.7 μg mL^{-1} (33.9 μM)	Mouse	[3]
Deguelin	*Mundulea sericea* A.Chev., 1925	*Haemonchus contortus*	Larval mortality after 72 h	IC_{50} = 14.8 μM	n.d.	[4,5]
Chlorogenic acid	*Tagetes filifolia* Lag., 1816	*Haemonchus contortus*	EHIA	LC_{50} = 248 μg mL^{-1}	n.d.	[6]
Caffeoyl and coumaroyl derivatives	*Acacia cochliacantha* (Mill.) Seigler & Ebinger	*Haemonchus contortus*	EHIA	†	n.d.	[7]
Epicatechin	*Persea americana*	*Haemonchus contortus*	LMIA	IC_{50} = 10 μg mL^{-1}	Goat	[1]
Oxytroside	*Gliricidia sepium* (Jacq.) Kunth ex Walp., 1842	*Cooperia punctata*	LDA and LEIA	100% inhibition of exsheathment at 2400 μg mL^{-1}	Calves	[8]
Isokaempferide	*Baccharis conferta* Kunth, 1818	*Haemonchus contortus*	EHIA	IC_{50} = 80 μg mL^{-1}	n.d.	[9]

Compound	**Plant species**	**Parasite species**	***In vitro* assay**	***In vitro* results**	***In vivo* model**	**Ref.**
Gallic acid	*Caesalpinia coriaria (Jacq.) Willd.*	*Cooperia* spp, *Haemonchus* spp., *Ostertagia* ssp., *Trichostrongylus* spp. and *Oesophagostomum* spp.	EHIA	100% inhibition at 1000 μg mL^{-1}.	n.d.	[10]
Procyanidin A2	*Alectryon oleifolius* (Desf.) S.T.Reynolds, 1987	cyathostomins	LMIA	IC_{50} = 12.6 μg mL^{-1}	n.d.	[11]

[1] Soldera-Silva et al., 2018; [2] Barrau et al., 2005; [3] Wangchuk et al., 2016; [4] Dilrukshi et al., 2017; [5] Preston et al., 2017; [6] Jasso Díaz et al. 2017; [7] Castillo-Mitre et al., 2017; [8] von Son-de Fernex et al., 2018; [9] Cortes-Morales et al., 2019; [10] García-Hernandez et al., 2019; [11] Payne et al., 2018. † At 1 mg mL^{-1} caffeic acid (98%), methyl caffeate (88%), methyl-p-coumarate (88%) and methylferulate (75%) show egg hatching inhibition. Additionally, p-coumaric acid/ferulic acid mixture and methyl ferulate/quercetin also showed 94% egg hatch inhibition.

1.4. SALT-TOLERANT PLANTS IN THE MEDITERRANEAN REGION

Salt-tolerant plants, which includes halophytes, are a group of terrestrial plants belonging to the extremophile group, which have salt tolerance as a major feature. Salt-tolerant plants have the ability to complete their life cycle in saline environments also characterized by other abiotic stressors like drought, flooding, ultraviolet exposure, and heavy metals accumulation (Nikalje et al., 2019). These locations include estuaries, salt marshes, dunes, rocky coasts and saline inland deserts (Ksouri et al., 2012a). In 1994, Le Houérou estimated that around 6,000 halophyte plant species occur worldwide, from which 1,100 inhabit the Mediterranean basin, with a wide range of tolerance levels to salinity.

The definition of salt-tolerant plants, particularly of halophytes, is not consensual, as some authors suggest that halophytes can grow well in salinity areas over 0.5% of NaCl concentration (Chapman, 1942) or that are able to tolerate at least 200 mM of NaCl (Flowers & Colmer, 2008). Likewise, others differentiate them into categories according to their preference for saline and non-saline habitats (Devi et al., 2019). However, the threshold of salt in the environment favoring the development of these three categories can be challenging to define. Recently, an online database of salt-tolerant plants was created, namely eHALOPH (http://www.sussex.ac.uk/affiliates/halophytes/), based on the records of Aronson (1989), which includes plants able to tolerate at least 80 mM of NaCl concentrations (approx. 8 dS m^{-1} of electrical conductivity; Santos et al., 2016). Therefore, in this work, the term "salt-tolerant" was adopted, in accordance with this broader definition accepted as an inclusion criterion in the eHALOPH database (Santos et al., 2016).

Salt-tolerant species include perennial and annual plants encountered among diverse ecological habitats, distributed in a wide range of botanical families. In the Mediterranean region, Amaranthaceae species are dominant, followed by Poaceae, Compositae, Caryophyllaceae, Leguminosae, Zygophyllaceae, Aizoaceae, Frankeniaceae, Tamaricaceae, Cyperaceae, Plantaginaceae, amongst others (Le Houérou, 1994; El Shaer & Attia-Ismail, 2015), comprising a variety of life forms, such as trees, shrubs, succulent plants, and seagrasses.

Located between land and sea, in maritime influenced environments, some species inhabit sand dunes, frequently exposed to salt spray (*e.g.*, *Calystegia soldanella* (L.) R.Br. 1810, *Medicago marina* L. 1753, *Armeria maritima* (Mill.) Willd., 1809*, Eryngium maritimum* L. 1753), whilst others are adapted to highly saline habitats, exposed to tides, such as saltmarshes (*e.g.*, *Atriplex halimus*, *Inula crithmoides* Dumort., 1827, *Limoniastrum monopetalum* and *Salicornia* L. 1753 spp.). Indeed, considering their ecological aspects, the latter generally tolerate seawater salinity levels, while, for example, *A. maritima and C. soldanella,* withstand a maximum salinity of 200 mM and 100 mM, respectively, according to the eHALOPH database (http://www.sussex.ac.uk/affiliates/halophytes/).

Depending on the habitat settings, these species evolved different structural, physiological and biochemical strategies to handle the high levels of NaCl in soil, which poses an abiotic challenge for most plants. Salinity tolerance mechanisms are intricate and not yet fully enlightened but generally comprise osmotic adjustment through accumulation of organic solutes, ion homeostasis, compartmentalization and export and/or excretion of sodium ions, coupled with the expression of salinity-stress related genes (*e.g.,* salt overly sensitive genes) and transcription factors (Flowers & Colmers, 2008; Balasubramaniam et al., 2023). Examples of these adaptation processes are salt secretion by Plumbaginaceae species, which possess specialized glands that excrete salt on the leaf surface (Grigore & Toma, 2016). Another evolutionary trait of these plants to withstand ecological stresses that trigger ROS production is their powerful antioxidant systems, reliant on the synthesis of several bioactive metabolites, such as enzymes, polyphenols, sterols, alkaloids, fatty acids and vitamins (Jaleel et al. 2009; Pirasteh-Anosheh et al., 2023).

A broad spectrum of potential applications of salt-tolerant species has attracted the attention of the scientific community and several industries. Emphasis has been given to saline agriculture for food and feed production: the possibility of several salt-tolerant species to be irrigated with brackish or seawater offers a significant advantage to glycophytic crops, having in mind the demand for novel food products due to a fast-growing world population paired with challenges like rising land degradation, soil salinization and climate change (Ventura et al. 2015; Bazihizina et al., 2024). Perhaps the most successful case is *Salicornia* spp.: In Portugal, *Salicornia* spp. is being cultivated

either in their natural environment, by Qampo™, in hydroponics, by Riafresh®, or in soil, by Salivitae, and commercialized either as a fresh vegetable or as sea salt. In addition, Riafresh® extended their commercial portfolio to other salt-tolerant species as fresh salty vegetables like *Mesembryanthemum crystallinum* L. 1753, *M. nodiflorum* L. 1753, *Plantago coronopus* L. 1753, *Crithmum maritimum* L. 1753, *Inula crithmoides* and *Cakile maritima* Scop., 1771. Other attractive economical utilizations of salt-tolerant plants comprise phytoremediation, as ornamental plants, as fodder resources and, finally, as sources of bioactive products (Manousaki & Kalogerakis, 2011; Ksouri et al. 2012a,b; Ventura et al., 2015; Lopes et al., 2023; Stanković et al., 2023). The vast ethnobotanical and ethnopharmacological descriptions on salt-tolerant plants (Ksouri et al., 2012a; Petropoulos et al. 2018) find support on the cumulative scientific evidence validating these plants as promising sources of added-value metabolites for a multitude of biotechnological applications, including food, feed, cosmetical and pharmaceutical industries (Custódio et al. 2012; Ksouri et al. 2008; 2009; 2012a,b; Lopes et al. 2016; 2023; Oliveira et al. 2016, 2018; Barreira et al. 2017; Pereira et al. 2017a,b,c; 2019; 2024; Rodrigues et al. 2014, 2017a,b, 2018; 2019; Castañeda-Loaiza et al., 2020a,b; Placines et al., 2020). Indeed, the growing biotechnological interest in the bioactives of these plants has driven the development and application of *in vitro* tissue culture methodologies, opening the venue to enhance the exploitation of their metabolites of interest (often extracted in low quantities) while avoiding environmental influences, sustainability concerns and cultivation issues and promoting species conservation, as comprehensively illustrated by Custódio and colleagues (2022).

Still, ethnoveterinary data on these plants is usually scattered in general ethnobotanical, ethnomedical, and ethnoveterinary surveys, and their potential applications in the context of veterinary sciences remains largely unexplored.

1.4.1. Ethnoveterinary uses of salt-tolerant plants

For millennia, edible salt-tolerant species have been included in local gastronomy as food products but also as livestock forage and fodder, particularly in seasonal supply shortages or as feed additional resources under drought conditions (El Shaer, 2010), and/or used for a panoply of therapeutic purposes (Ksouri et al. 2012a,b). Additionally, numerous species exhibit medicinal properties, sustained by scientific evidence on the

presence of bioactive metabolites (Ksouri et al., 2021a,b). Although the majority of reports on salt-tolerant plants medicinal properties focused on human therapeutic uses, these may also be useful in veterinary practices and animal health management, keeping in mind that traditional human and veterinary knowledge frequently overlap (McCorkle, 1986; Pieroni et al., 2006; Benítez et al., 2012; Miara et al. 2019). However, information on their traditional verinary uses is usu- ally dispersed in ethnobotanical, ethnomedical and ethnoveterinary surveys. Despite discussions around the use as forage and fodder crops for livestock (Le Houérou, 1994; Tag El-Din, 2012; Norman et al., 2013; El-Shaer and Attia-Ismail, 2015; Attia-Ismail, 2018; Abd El-Hack et al., 2018), the potential veterinary applications of these plants remain largely underexplored.

A systematic review carried out under the scope of this dissertation compiled salt-tolerant species from the Mediterranean region used as animal feed/fodder, nutraceuticals and/or as phytotherapeutics (Suppl. data – Table S1; Oliveira et al. 2021), and the main findings and discussion regarding ruminant animals are presented in the following sub-sections.

Animal feed and fodder

Eight reports are unambiguously linked to animal feed, particularly the species *Atriplex halimus*, *Foeniculum vulgare* Mill., 1768, *Plantago major* L. 1753, *Cynodon dactylon* (L.) Pers. 1085, *Trifolium* L. 1753 sp. and *Setaria* P.Beauv. sp., either as feed, fodder, or food integrator. In agreement, some of these species are currently recognized animal feed resources, being its nutritional aspects summarized in the online platform of the Feedipedia project (www.feedipedia.org; Heuzé et al., 2015a,b; 2019).

Animal feed is defined by FAO and IFIF (2010) as "*any single or multiple materials, whether processed, semi-processed or raw, which is intended to be fed directly to food-producing animals"*. In 1994, Le Houérou suggested some salt-tolerant plants from the Mediterranean Basin to be used as forage and fodder crops for livestock animals, including *Hedysarum carnosum* Desf. 1799, *Elymus elongatus* (Host) Runemark, *Cynodon dactylon*, *Phalaris* L. 1753 spp., *Trifolium* spp., *Lotus* L. 1753 spp., *Lolium rigidum* Gaudin 1811, *Medicago* L. 1753 sp. and *Melilotus* Mill. spp., amongst others.

More recently, the perspective of using salt-tolerant plants as feed resources for livestock production, particularly for ruminants, has been discussed in different works (El

Shaer, 2010; Tag El-Din, 2012; Norman et al., 2013; Attia-Ismail, 2018). The authors highlight the drawbacks that must be considered when exploring salt-tolerant species as animal feed, which are mainly related to its high salt contents, low palatability, low metabolizable energy, and the presence of anti-nutritional metabolites, such as alkaloids, saponins, tannins, flavonoids, oxalates and organic acids (Le Houérou, 1994; Tag El-Din, 2012; Norman et al., 2013; Abd El-Hack et al., 2018; Attia-Ismail, 2018).

A paradigm shift developed over the last decades led to the perception of these anti-nutritional compounds as plant bioactive metabolites, initiating discussions on how they can positively impact animal health, performance and quality of derived-food products (Mueller-Harvey, 2006; Rochfort et al., 2008; Patra & Saxena, 2009; Zhong & Zhou, 2013; Hoste et al., 2015; Olagaray & Bradford, 2019). Still, achieving beneficial outcomes depends on diverse factors such as the concentration in the animal diet, chemical structure, diet composition, animal species and physiological status (Patra & Saxena, 2009; Mueller-Harvey et al., 2018). Nevertheless, this opens a new awareness into salt-tolerant species phytochemistry that can be exploited to be used advantageously in veterinary science.

Nutraceutical plants

Despite reports of the use of salt-tolerant species as animal feed and fodder, few are indicative of further use as nutraceuticals, and mainly species of the *Trifolium* genus have been traditionally used as fodder (Di Novella et al., 2013) and nutraceutical fodder for cattle (Pieroni et al., 2003). The scarcity of nutraceutical uses among salt-tolerant species may be related to the fact that the concept of "*nutraceutical*" is relatively recent, and therefore plants would not be described under such vocable. For example, *C. dactylon* is used as animal feed, and fodder for ruminants (Di Novella et al., 2013; revised by Lucchetti et al. 2019) and rhizomes have been described as used as fodder alleviating intestinal inflammations and as a diuretic for sheep (revised by Pieroni et al., 2004). Although not mentioned as a nutraceutical, the use of such species could be perceived as possibly combining feed and pharmacological effects. Other examples are the use of *F. vulgare* fruits as fodder to improve the milk supply (galactagogue) of sheep (Pieroni et al. 2006) or leaves put in cattle feed to counteract abdominal bloat (Lucchetti et al. 2019).

Similarly, whole plants without roots of *Phragmites australis* (Cav.) Trin. ex Steud 1841. are given as forage to treat diarrhea in bovines (Uncini Manganelli et al. 2001).

Herbal remedies (= phytotherapeutics)

The attribution of the respective ATCvet codes (Anatomical Therapeutic Chemical (ATC) classification system for veterinary medicines; https://www.whocc.no/atcvet/atcvet/) to each ethnoveterinary report with therapeutic indication revealed that three categories account for more than three quarters of the total: alimentary tract and metabolism (QA; n = 75, 33.9%), dermatological (QD; n = 53, 24.0%) and genitourinary system and sex hormones (QG; n = 41, 18.6%; Figure 1.6; Oliveira et al. 2021), in agreement with results obtained in different ethnobotanical, -pharmacological and -veterinary surveys throughout the Mediterranean region (Bonet & Vallés, 2007; Cornara et al., 2009; Akerreta et al., 2010; González et al., 2011; Carrió et al., 2012; Ali-Shtayeh et al., 2016; Erarslan & Kültür, 2019).

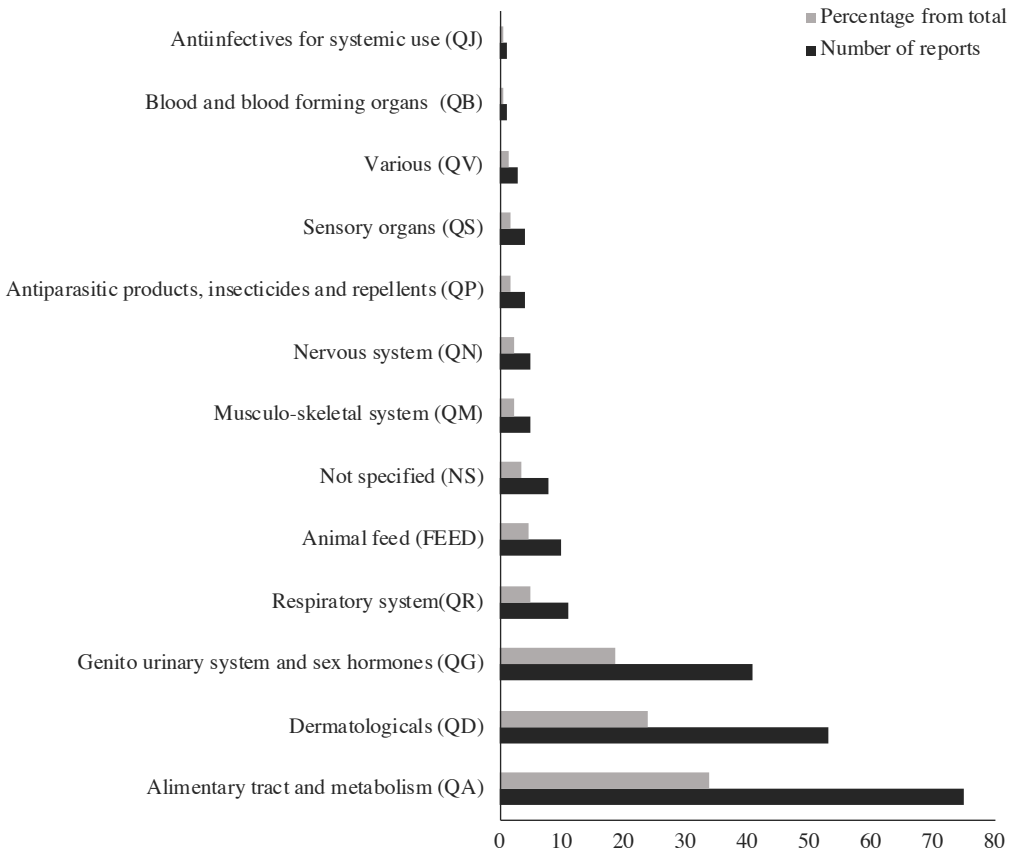

Figure 1.6 Distribution of the 221 ethnoveterinary reports among the different ATCvet categories, represented by the total number of reports and by percentage, for each group (published in Oliveira et al. 2021).

Within these three major groups, undoubtedly, five species stand out, with a minimum of 5 reports in at least one of the three most mentioned categories (QA, QD, QG), namely *Hordeum vulgare* L. 1753, *P. lentiscus*, *Dittrichia viscosa* (L.) Greuter 1973, *F. vulgare* and *P. major* (Table 1.5).

Table 1.5 Number and respective percentage of ethnoveterinary reports (EVR) in relation to total in square brackets, for the three most relevant ATCvet codes, organized by a descendent order of the most cited salt-tolerant plant species. Species with more than 5 EVR in at least one of the ATCvet categories are presented in bold. QA, Alimentary tract and metabolism; QD, Dermatologicals; QG, Genitourinary system and sex hormones (published in Oliveira et al. 2021).

Plant species	QA	QD	QG
***Hordeum vulgare* L.**	**17 [22.7]**		**13 [31.7]**
***Pistacia lentiscus* L.**	**12 [16.0]**	**4 [7.5]**	**3 [7.3]**
***Dittrichia viscosa* (L.) Greuter**	**7 [9.3]**	**7 [13.2]**	**3 [7.3]**
***Foeniculum vulgare* Mill.**	**6 [8.0]**		**8 [19.5]**
***Plantago major* L.**	**1 [1.3]**	**11 [20.8]**	**1 [2.4]**
Eucalyptus camaldulensis Dehnh.	5 [6.7]	3 [5.7]	
Plantago lanceolata L.	2 [2.7]	4 [7.5]	2 [4.9]
Drimia maritima [L.] Stearn	3 [4.0]	4 [7.5]	
Achillea millefolium L.	3 [4.0]	4 [7.5]	
Peganum harmala L.	2 [2.7]	2 [3.8]	1 [2.4]
Atriplex halimus L.	2 [2.7]		2 [4.9]
Calotropis procera [Aiton) Dry and.	2 [2.7]	1 [1.9]	
Cynara cardunculus L.	2 [2.7]		1 [2.4]
Arundo donax L.	2 [2.7]		1 [2.4]
Cynodon dactylon (L.) Pers.	2 [2.7]		1 [2.4]
*Anabasis articulata (*Forssk.) Moq.		2 [3.8]	
Daucus carota L.	2 [2.7]		
Brassica oleracea L.		2 [3.8]	
Juncus maritimus Lam.			2 [4.9]
Althaea officinalis L.		2 [3.8]	
Lycium shawii Roem. & Schult.		2 [3.8]	
Verbena officinalis L.		1 [1.9]	1 [2.4]
Haloxylon scoparium Pomel		1 [1.9]	
Phoenix dactylifera L.		1 [1.9]	
Raphanus raphanistrum L.		1 [1.9]	
Acacia nilotica L. Delile	1 [1.3]		
Phillyrea latifolia L.	1 [1.3]		
Plantago coronopus L.			1 [2.4]
Phragmites australis (Cav.) Trin. ex Steud.	1 [1.3]		
Rumex crispus L.		1 [1.9]	
Rumex sp.	1 [1.3]		
Rubia tinctorum L.			1 [2.4]
Populus sp.	1 [1.3]		
Chenopodium ambrosioides L.			
Malva arborea (L.) Webb & Berthel.			
Total EVR (by category)	**75 [100]**	**53 [100]**	**41 [100]**

It is not surprising that *H. vulgare*, *P. lentiscus*, *F. vulgare*, and *P. major* were amongst the most quoted species, since these have a long history of traditional uses for humans and animals, either as crops (*e.g.*, *H. vulgare*) or for its medicinal value (*e.g., P.*

lentiscus; Landau et al., 2014). In addition, some are included in the European Pharmacopoeia (Ph. Eur.), namely *F. vulgare* (fennel) and *P. lentiscus* mastic resin. Moreover, extensive information on their phytochemistry and bioactive properties is available, sustaining some of its ethnoveterinary uses, as comprehensively reviewed by others, for *P. lentiscus* (Bozorgi et al., 2013; Landau et al., 2014), *P. major* (Gonçalves & Romano, 2016; Adom et al., 2017), *F. vulgare* (Rather et al., 2016) and *D. viscosa* (Barrero et al., 2008).

Nevertheless, these species have relatively low to moderate salt tolerance. For example, according to the eHALOPH database, *H. vulgare* withstands a maximum salinity of 200 mM NaCl, while the salt tolerance of *F. vulgare* is lower (100 mM NaCl). Similarly, *P. lentiscus* was found to tolerate and accumulate salt, supporting its wide distribution along the Mediterranean coastal areas (Barazani & Golan-Goldhirsh, 2009). Moreover, recent studies emphasize the moderate salinity resistance of *P. major* and *Plantago lanceolata* L. 1753, although being less salt-tolerant than other related species such as *Plantago maritima* L., 1753 and *P. coronopus* (Al Hassan et al., 2016; Izadi-Darbandi & Mehdikhani, 2018).

In 2016, the eHALOPH database included 1457 records of salt-tolerant plants, from which around 23% represent highly salt-tolerant species (>200 mM NaCl; Santos et al., 2016). However, only few species from the former group were identified in this work, such as *Calotropis procera* (Aiton) W.T.Aiton, 1811, *Atriplex halimus*, *Acacia nilotica* (L.) Willd. ex Delile, *Phillyrea latifolia* L. 1753, *P. coronopus*, *Elymus repens* (L.) Gould 1947 and *Phragmites australis* (Cav.) Trin. ex Steud., 1841. Highly salt-tolerant species have been narrowly mentioned in ethnoveterinary reports, perhaps due to the habitat where they are encountered or for having stricter ecological requirements. These, *i.e.*, highly saline environments, would be of difficult access or less explored by the local communities. In fact, only the recent work of Miara and colleagues (2019) refers to plants growing in salty soils, namely in the Algerian steppe (*e.g.*, *A. halimus*). Nevertheless, some have traditional human medicine records and have been investigated for their diverse chemical composition, including phytochemical content and bioactive properties (Küpeli et al., 2006; Abdel-Wahhab et al., 2008; Ksouri et al., 2009, 2012b; Chaturvedi et al., 2012; Lopes et al., 2016; Rodrigues et al., 2014, 2018). Having in mind the rising attention in using plant bioactive metabolites (tannins, flavonoids, saponins), further

investigations on these underexplored salt-tolerant species can unravel an added-value resource to improve ruminant health and productivity. As an example, numerous highly salt-tolerant species are rich sources of phenolic compounds, like tannins and flavonoids (e.g., *Limoniastrum monopetalum* L. and *C. edulis*; Custódio et al., 2012; Lopes et al., 2016; Castañeda-Loiaza et al., 2020a,b), which may pose an opportunity to explore its anthelmintic and anti-bloat properties (Hoste et al., 2015), effects on the ruminal biohydrogenation and fermentation patterns (Patra and Saxena, 2009) and the management of oxidative-related disorders (Olagaray & Bradford, 2019).

1.4.2. Salt-tolerant species selection

In 2012, the XtremeBio project (PTDC/MAR-EST/4346/2012) initiated investigations on the chemical and bioactive properties of salt-tolerant plants from the Algarve coastaline, Southern Portugal, allowing the identification and biotechnological prospection of several species. From this initial list of plants, and under the framework of the GreenVet project (GreenVet-ALG-01-0145-FEDER-028876), nine plant species were selected for this dissertation. The subsequent sub-sections intend to introduce the selected plants as well as summarize relevant bibliographic information on its ethnobotanical uses, phytochemical content, and bioactive properties, which altogether echoed on the final plant selection verdict, alongside with availability and access of wild plant biomass.

1.4.2.1. Pistacia lentiscus L., 1753

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Sapindales; Anacardiaceae; *Pistacia* L. (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

Pistacia lentiscus, also known as lentisk in English and ‘’alfostigueiro’’, ‘’almecegueira’’, ‘’almestigueiro’’, “lentisco-verdadeiro”, ‘’lentisco’’, ‘’daro’’, ‘’árvore-do-mástique’’ or “aroeira” in Portuguese, is an evergreen shrub distributed along the Mediterranean region (Henning & Raab-Straube, 2016; Castroviejo et al., 2015; Figure 1.7-A). The flowering period of this dioecius species, *i.e.*, has male and female individuals, occurs simultaneously in early spring, with male individuals having between 5-6 bracteoles while female flowers hold 4-5 (Martínez-Pallé & Aronne, 2000; Milla et al., 2006; Castroviejo et al., 2015; Figure 1.7-B). It is common for flowers to fail on developing into fruits, accounting its low reproductive success to this issue (Martínez-Pallé & Aronne, 2000). After flowering, fruit development occurs in summer-autumn, with red fruits becoming almost black as they ripen (Figure 1.7-C; Martínez-Pallé & Aronne, 2000; Milla et al., 2006).

Ethnobotanical uses

P. lentiscus resin (mastic resin) is listed in the European Pharmacopoeia (Ph.Eur.). Among plentiful ethnobotanical descriptions, its ethnoveterinary uses are highlighted: aerial parts, fruits, leaves, and wood are used traditionally against gastrointestinal disorders in livestock, including bloat in cattle, constipation, flatulence, and diarrhea, leaves are used as a digestive for ovines and aerial parts as an appetizer for ruminants (Suppl. data – Table S1; Oliveira et al., 2021).

Phytochemical content and bioactive properties

Extensive phytochemical research has been summarized on lentisk essential oils, organic extracts, and mastic resin, highlighting its polyphenolic (*e.g.,* flavonoids and tannins) and terpenoid content coupled with several *in vitro* and *in vivo* biological activities ascribed to this species like antioxidant, antimicrobial, anti-inflammatory and anthelmintic (Bozorgi et al., 2013; Rauf et al., 2017).

Due to wide scientific validation of its ethnoveterinary claims, particularly on anthelmintic effects linked to its high content on polyphenols, this species was used as reference plant along this work.

A

B

C

Figure 1.7 Aerial organs of *Pistacia lentiscus*: **A**) watercolour illustration; **B**) details of inflorescences; **C**) and fruits (**B**; illustration and photos by the author).

1.4.2.2. Helichrysum italicum subsp. picardii (Boiss. & Reut.) Franco 1984

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Asterales; Asteraceae; *Helichrysum* Mill. 1754 (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

The aromatic shrub *H. italicum picardii* is also known as "everlasting" in English and "perpétua-das-areias" or "erva do caril" in Portuguese (Jardim Botânico UTAD, 2025), the latter inspired by its curry smell (Figure 1.8). *H. italicum* plants occur in dry, sandy, and stone areas in the Mediterranean area, but *H. italicum picardii* is rather restricted to France, Portugal, Italy, Spain and Morocco (Viegas et al., 2014; Greuter, 2006a). Similarly to its related subspecies, its vegetative period starts in autumn (after the first rainfall events), and shoot elongation begins in spring (Cesaraccio et al., 2004). Afterward, flowering rapidly occurs in early summer (Figure 1.8-B), and the plant ceases vegetative activity in the mid-summer period (Cesaraccio et al., 2004).

Ethnobotanical uses

Above-ground organs are used for flavoring in local gastronomy (Guarrera, 2006), while medicinal claims of *H. italicum*, include the treatment of gastrointestinal complaints and parasitic intestinal infections, dermatologic, inflammatory, and respiratory disorders (Viegas et al., 2014). Moreover, aerial parts are used to stimulate rumination in bovines and to improve milk quality in sheep (Uncini-Manganelli et al., 2001; Viegi et al., 2003; Guarrera, 2005). The subspecies *H. italicum picardii* is scarcely mentioned, though its essential oil is used to treat dermatomycosis in Portugal, as reviewed by Viegas et al., (2014).

Phytochemical content and bioactive properties

Phytochemical investigations on the infusions and decoctions of *H. italicum picardii* revealed that aerial organs (stems, leaves and flowers) are enriched in phenolic compounds, particularly caffeoylquinic and dicaffeyolquinic acids, displaying antioxidant and anti-diabetic properties (Pereira et al., 2017b).

A

B

C

Figure 1.8 Aerial organs of *Helichrysum italicum* subsp. *picardii*: **A**, watercolour illustration; **B**, details of flowers in summer; **C**, leaves in spring (illustration and photos by the author).

1.4.2.3. Inula crithmoides L. (syn. *Limbarda crithmoides* Dumort. 1827)

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Asterales; Asteraceae; *Limbarda* Adans. 1753 (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

Inula crithmoides, also known as "golden samphire" in English and "campana-da-praia", ''mardoneira rasteira'', "madorneira-bastarda" in Portuguese, is a perennial species with succulent leaves grows in coastal saltmarshes of the Mediterranean basin (Figure 1.9; Greuter, 2006b). The flowering period occurs during summer, when small yellow flowers are observed, followed by seed ripening in autumn (Figure 1.9-C).

Ethnobotanical uses

Leaves are edible, being widely used in Mediterranean gastronomy, mixed in salads, and appreciated for their salty flavor (Zurayk & Baalbaki, 1996; Scherrer et al., 2005; Guarrera, 2006; Petropoulos et al., 2018). Its fodder potential is emphasized by field observations of goats grazing on this plant along the Lebanese coast (Zurayk & Baalbaki, 1996). A panoply of traditional uses is given to *Inula* sp. (Seca et al., 2014); however, few concerns *I. crithmoides*. In Egypt, it is valued for its medicinal value (Shaltout & Ahmed, 2012). In ethnoveterinary practices, *Inula* sp. is used to treat respiratory diseases of equines, and *I. viscosa* (*syn. Dittrichia viscosa*) is broadly used for the treatment of gastrointestinal ailments (diarrhea, flatulence, helminths), inflammatory and dermatological disorders of ruminants, and as an appetizer (Suppl. Data, Table S1; Oliveira et al., 2021).

Phytochemical content and bioactive properties

Organic extracts have high contents of phenolics, especially caffeoylquinic, dicaffeyolquinic acids, and quercetin, exhibiting antioxidant, antibacterial, anti-tyrosinase (Jallali et al., 2014; 2020; Jdey et al., 2017) and anti-mutagenic activities (Abdel-Wahhab et al., 2008). Essential oils are rich in monoterpene and sesquiterpene hydrocarbons and phenols (thymol derivatives), with antioxidant properties (Giamperi et al., 2010; Fontana et al., 2014) and antiproliferative properties (Adorisio et al., 2020). In

addition, it is a good source of vitamin B1, B6, lutein, *β*-carotene and diverse phenolic structures.

Figure 1.9 Aerial organs of *Inula crithmoides*: **A**, watercolour illustration; **B**, details of flowers in autumn; **C**, leaves in spring (illustration and photos by the author).

1.4.2.4. Calystegia soldanella (L.) R.Br. 1810 (syn. *Convolvulus soldanella* L. 1753)

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Solanales; Convolvulaceae; *Calystegia* (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

Calystegia soldanella, also known as "sea bindweed" and "morning glory" in English or "couve-do-mar", ''couve marítima'', ''soldanela'', ''soldanela bastarda'', ''soldanella-do-litoral'' or ''versa-marinha'' in Portuguese, is a perennial glabrous plant that thrives in sandy soils of beach dunes of temperate regions (psammophile; Castroviejo, 2012; Figure 1.10). These herbs have procumbent stems, long petiolate-shaped leaves (Figure 1.10-C), and solitary pinkish pedunculated inflorescences with bracts, which arise during spring (Castroviejo, 2012; Figure 1.10-C).

Ethnobotanical uses

In Portugal, extracts are reported as used to treat hydropsy, paralysis, rheumatism, and scurvy (reviewed by Gaspar, 1999). Additional uses include diuretic, irritant, laxative and purgative, anthelmintic and to treat fever (U.S. Department of Agriculture).

Phytochemical content and bioactive properties

C. soldanella is particularly studied for its resin glycosides, some with antiviral properties (Ono, 2017) and alkaloids (Asano et al., 2001). Apart from these, flavonoids have also been identified in its leaves (Murai et al., 2015) and flowers (Tatsuzawa et al., 2004), and a pentasaccharide macrolatone was isolated from its roots (Gaspar, 1999). Additional bioactive properties ascertained to *C. soldanella* include *in vitro* antiproliferative (Lee et al., 2017) and anti-inflammatory effects (Kim et al., 2004).

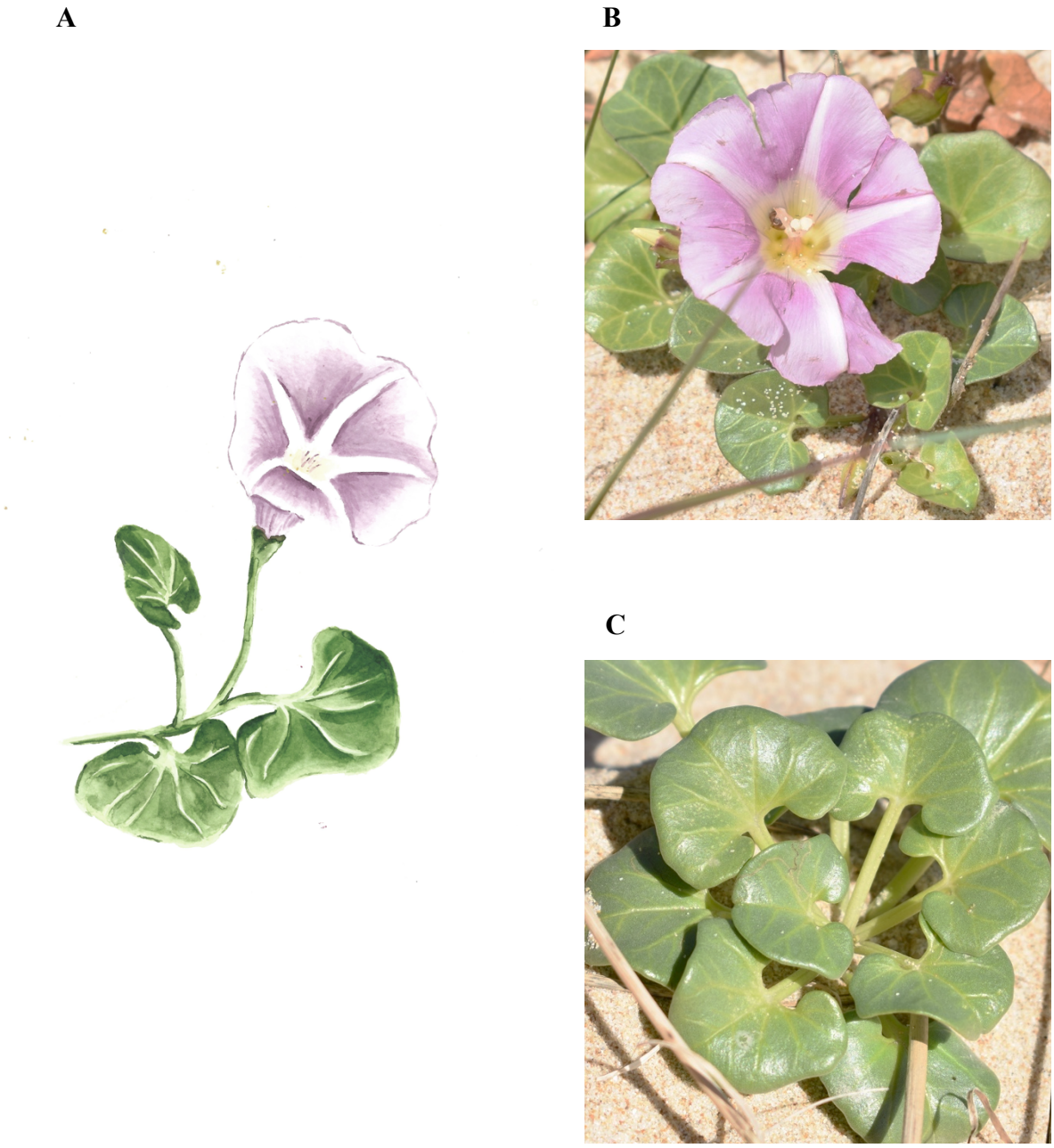

Figure 1.0 Aerial organs of *Calystegia soldanella*: **A**, illustration; **B**, details of flowers during spring; **C**, details of leaves in autumn (illustration and photos by the author).

1.4.2.5. Cladium mariscus L. Pohl. 1809

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Liliopsida; Poales; Cyperaceae; *Cladium* P.Browne (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

Cladium mariscus is a grass-like perennial herbaceous species, also known as "sawgrass" or "sawsedge", distributed across Europe and along the Mediterranean area (Jiménez-Mejias & Luceño, 2011) in low to moderate saline habitats (Gerdol et al., 2018). *C. mariscus* lengthy and everlasting leaves have characteristic saw-shaped margins and inflorescences rise above leaves during summer, yielding fascicles that are composed of around 10-25 spikelets (Castroviejo et al., 2008; Figure 1.11).

Ethnobotanical uses

In Britain, historical descriptions of *C. mariscus* report their use mostly in thatching and as a fuel (Rowel, 1986). In traditional Egyptian medicine, a decoction of *C. mariscus* is applied to manage symptoms of cold, renal pain, and gastrointestinal colics (AbouZid & Mohamed, 2011; AbouZid, 2015).

Phytochemical content and bioactive properties

The analysis of nutlets, rhizomes and culms of *C. mariscus* collected in South Africa showed that nutlets are not a rich source of protein or fat but have a high carbohydrates content (Sievers, 2015). *C. mariscus* aerial parts 80% acetone aqueous extracts exhibited high total polyphenol content, particularly total flavonoids and tannins, and exhibit radical scavenging properties (Lopes et al., 2016).

A

B

C

Figure 1.11 Aerial organs of *Cladium mariscus*: **A**, watercolour illustration; **B**, details of inflorescences in summer; **C**, leaves in summer (illustration and photos by the author).

1.4.2.6. Medicago marina L. 1753

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Fabales; Fabaceae; *Medicago* L. 1753 (abbreviated lineage according to Schoch et al., 2020).

General botanical aspects and distribution

Medicago marina L., also known as sea medick in English, or "erva-cordeira", "erva-das-areias", "luzerna-das-areias", ''erva-do-perdão'' or "melga-da-praia" in Portuguese, is a perennial herb inhabiting littoral dunes and sea gravels (psammophile; Talavera et al., 1999). *M. marina* is fully covered in whitish trichomes and has robust stems that give rise to abundant leaves with obovate to flabellate shaped leaflets (Talavera et al., 1999; Figure 1.12). Blooming of lemon-yellow flowers occurs during spring (Figure 1.12-B), while robust spiny fruits emerge afterward, during summer (Figure 1.12-C).

Ethnobotanical uses

Medicago sp. is used in Egypt for its medicinal properties and livestock grazing (Bidak et al., 2015), and for swelling, labor complications as retained placenta and to increase milk secretion, in veterinary traditional practices in Turkey (Erarslan & Kültür, 2019; Suppl. Data, Table S1; Oliveira et al., 2021).

Phytochemical content and bioactive properties

Concerning its secondary metabolites, monoterpenes, sesquiterpenes and phenylpropanoids were quantified in essential oils of this psammophile aerial parts, collected in two development stages in Italy (Flamini et al., 2003). More recently, triterpenic saponins from *M. marina* leaves and roots were deeply characterized (Tava et al., 2020).

A

B

C

Figure 1.12 Aerial organs of *Medicago marina*: **A**, watercolour illustration; **B**, details flowers in spring; **C**, details fruits in summer (illustration and photos by the author).

1.4.2.7. Plantago coronopus L. 1753

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Lamiales; Plantaginaceae; *Plantago* L. (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

P. coronopus is also known as "buckshorn plantain" in English and ''corno-de-veado'', ''erva-das-pulgas'', ''estrela-mar'', ''galapito'', ''guiabelha'', ''megabelha'', "diabelha", amongst others, in Portuguese (Castroviejo et al., 2009). These herbaceous plants, being annual or biannual, grow on saline or trampled coastal habitats of the Mediterranean region (Castroviejo et al., 2009; Figure 1.14). Pinnatifid leaves have a lanceolate to linear shape, flat, often dentate, and forme a rosette in the bottom, where cylindrical flowering spikes rise vertically during spring-summer (Castroviejo et al., 2009).

Ethnobotanical uses

P. coronopus is used in salads in European gastronomy mainly for its nutty taste and crisp texture (Heimler et al., 2007). In traditional animal practices, a wide range of therapeutic and nutritional uses are reported for *Plantago* species (Suppl. Data – Table S1; Oliveira et al., 2021): for example, *P. lanceolata* and *P. major* whole plant are used as feed for ruminants while leaves are largely used against dermatological ailments, including external parasites infestations. Similarly, *P. coronopus* is used as fodder for swine and to aid birth in caprines, and a tisane of leaves is used as a parasiticide for poultry (Suppl. Data, Table S1; Oliveira et al., 2021).

Phytochemical content and bioactive properties

Roots, leaves, and flowers extracts are rich in phenolic compounds (Pereira et al., 2017c) and iridoids were detected in its aerial parts (Janković et al., 2012). Cytotoxic, anti-inflammatory and anti-radical properties have been reported (Rodrigues et al., 2014; Pereira et al., 2017c). Additionally, leaves are a good source of minerals and amino acids (Pereira et al., 2017c).

A

B

C

Figure 1.13 Aerial organs of *Plantago coronopus*: **A**, watercolour illustration; **B**, in spring; **C**, in autumn (illustration and photos by the author).

1.4.2.8. Limoniastrum monopetalum (L.) Boiss., 1848

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Plumbaginaceae; *Limoniastrum* Fabr., 1759 (abbreviated lineage according to Schoch et al., 2020).

Distribution and general characteristics

Limoniastrum monopetalum is a highly salt-tolerant perennial shrub growing up in swamps, saltmarshes, sandy soils and rocky coastal regions of the Mediterranean region (Castroviejo et al., 1990; Figure 1.14). This shrub erect branches are covered with obovate-lanceolate silvery green leaves and pentameric pink/purpleish flowers emerge during spring, arranged in branched spiciform inflorescences (Castroviejo et al., 1990; Figure 1.14-B). A characteristic adaptation strategy used by *L. monopetalum* to counteract salinity is the presence of salt glands, which secrete salt crystals that accumulate in the leaf's surface (Akoumianaki-Ioannidou et al., 2015; Figure 1.14-C).

Ethnobotanical uses

In Tunisia, leaf and gall infusions are used to treat dysentery of infectious origin, including against parasites causing painful and bloody diarrhea (Chaieb & Boukhris, 1998).

Phytochemical content and bioactive properties

The fodder potential of this species is underlined due to its nutritive content (Neves et al., 2007; Zahran & El-Amier, 2013; Vizzeto-Duarte et al., 2019). High contents of tannins and flavonoids were identified in stems, leaves, and flowers extracts of *L. monopetalum* (Ksouri et al., 2008; Trabelsi et al., 2010; Bouzidi et al., 2016; Lopes et al., 2016). Gallic acid, vanillic acid, and quercetin are the phenolic compounds quantified in higher amounts among the different organs (Trabelsi et al., 2012). Regarding biological activities, antioxidant properties are reported (Debouba et al., 2013; Lopes et al., 2016) as well as antimicrobial effects (Trabelsi et al., 2010). Nevertheless, variations on the phenolic composition and antioxidant capacity of *L. monopetalum* extracts occur depending on the solvent used (Ksouri et al., 2008; Trabelsi et al., 2010) and studied organs (Trabelsi et al., 2012).

1

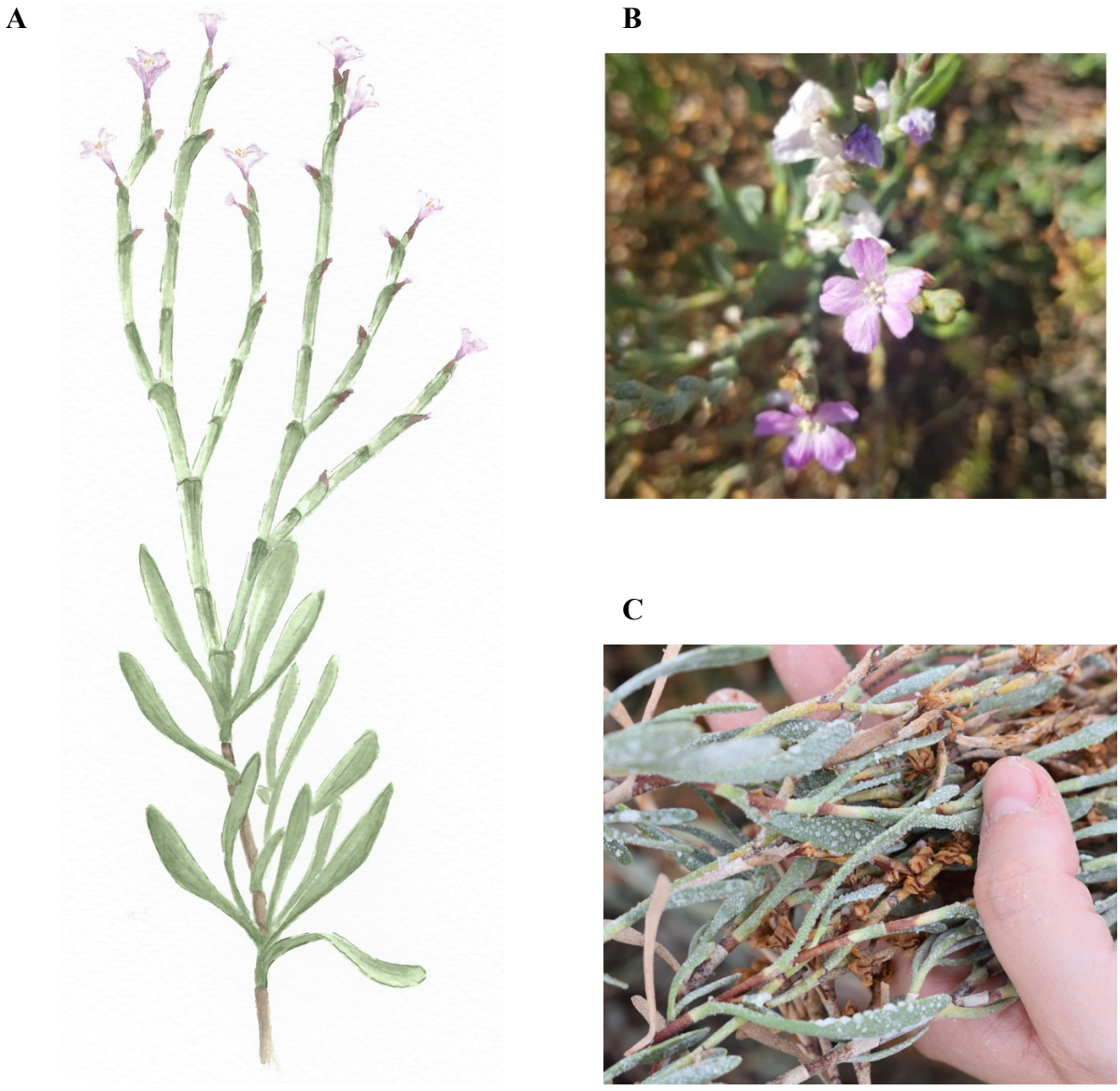

Figure 1.14 Aerial organs of *Limoniastrum monopetalum*: **A**, watercolour illustration; **B**, details of flowers during spring; **C**, details of salt crystals on leaf's surface (illustration and photos by the author).

1.4.2.9. Crucianella maritima L. 1753

Taxonomic classification

Eukaryota; Viridiplantae; Streptophyta; Magnoliopsida; Gentianales; Rubiaceae; *Crucianella* L. 1753 (abbreviated lineage; Schoch et al., 2020).

Distribution and general characteristics

Crucianella maritima, also known as "granza-da-praia" or "rubia-da-praia" in Portuguese, is an annual subshrub occurring in coastal dunes of the Mediterranean area (Castroviejo et al., 2007; Marhold, 2011; Figure 1.15). *C. maritima* has sharp and ovate-lanceolate shaped leaves in whorls, rising from woody stems, and small yellow pentameric sessile flowers emerge during spring (Castroviejo et al., 2007; Figure 1.15-B, C).

Ethnobotanical uses

Crucianella maritima is scarcely mentioned in ethnobotanical studies, only being reported to be used generally for medicinal purposes in coastal areas of Egypt (Bidak et al., 2015).

Phytochemical content and bioactive properties

Secondary metabolites identified in *C. maritima* include flavonoids with antioxidant and antimicrobial activities (Sabri et al., 1988; Badr, 2008), iridoids (Venditti et al., 2014), and anthraquinones with antibacterial properties (El-Lakany et al., 2004; Badr, 2008).

1

A

B

C

Figure 1.15 Aerial organs of *Crucianella maritima*: **A**, watercolour illustration; **B**, during spring; **C**, in winter (illustration and photos by the author).

1.5. OBJECTIVES

The main goal of this dissertation is to explore and valorize Mediterranean salt-tolerant species as sources of added-value products for the management of nematode parasitic infections in ruminant animals. Though multiple ethnobotanical descriptions and biochemical investigations on salt-tolerant species points towards biotechnological applications, the usefulness of their biological and chemical assets for the veterinary parasitology field has not yet been investigated. Additionally, most works narrow the worth of prospected plant species to target active metabolites, underestimating the sphere of opportunities available midway for a sustainable exploitation of the plant biomass. Thereof, under the framework of this dissertation, the following scientific questions were addressed:

a) Can these plants be considered nutraceutical plants?

b) Are these plants rich sources of bioactive phenolic metabolites of nutraceutical or phytotherapeutic interest?

c) Are the plant-derived products able to disrupt the biological cycle of gastrointestinal nematodes?

d) Are phenolic compounds involved in the anthelmintic effects?

e) Does seasonality impact the nutraceutical or phytotherapeutic value of these species?

f) Do *in vivo* trials sustain the interest for further veterinary applications?

SUPPLEMENTARY FILES

Table S1 Ethnoveterinary reports (EVR) of Mediterranean salt-tolerant species (Published in Oliveira et al., 2021, as Additional file 1). NS – Not Specified.

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Acacia nilotica L. Delile	Caprines	Digestive	Fruits, Leaflets	Decoction, Internal	Egypt	Pieroni et al. 2006
Achillea millefolium L.	NS	Stomach ache	Inflorescence	Infusion, Internal, Dry	Spain	Akerreta et al. 2010
	NS	Digestive	Aerial part	Infusion	Italy	Dei Cas et al. 2015
	NS	Diuretic	Leaves	Decoction	Italy	Idolo et al. 2010
	Bovines	Scabies	Inflorescence	Poultice of flowers and brandy, topical application	Italy	Menale and Muoio 2014
	Ovines	Scabies	Inflorescence	Poultice of flowers and brandy, topical application	Italy	Menale and Muoio 2014
	Equines	Scabies	Inflorescence	Poultice of flowers and brandy, topical application	Italy	Menale and Muoio 2014
	Bovines	Diarrhoea	Flowered aerial part	Tea, dried and sold	Albania	Pieroni et al. 2014
	Bovines	Anti-diarrhoeal, intestinal anti-inflammatory/antiinfective agents	Flowered aerial part	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Skin and wound	NS	NS	Italy	Revised by Viegi et al. 2003
	Bovines	Wound healing	Leaves	Ointment, external	Italy	Uncini Manganelli et al. 2001
	NS	Digestive	Flower	Infusion	Italy	Vitalini et al. 2009
	Bovines	Digestive	Flower, Leaves	Infusion	Italy	Vitalini et al. 2013
Aloe vera (L.) Burm. f.	NS	Bone reinforcer	Leaves	Direct application, External	Spain	Carrió et al. 2012
Althaea officinalis L.	NS	Sore-throat	Root	Vapour inhalation, External, Fresh	Spain	Akerreta et al. 2010
	NS	Wounds	Root	Liniment (with *Bryonia cretica* or *Umbilicus rupestris*), External	Spain	Carrió et al. 2012

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Althaea officinalis L.	Equines	Oral inflammations	Whole plant	Wrapped in linen and placed in the oral cavity, External	Morocco	Pieroni et al. 2006
	Bovines	Ailments of the sensory organs	NS	NS	Italy	Revised by Viegi et al. 2003
	Bovines	Skin and wound	NS	NS	Italy	Revised by Viegi et al. 2003
	Bovines	Anti-inflammatory	Root	Decoction applied in the inflammed withers where the yoke rubs	Italy	Uncini Manganelli et al. 2001
Amaranthus sp.	NS	Antiparasitics	NS		Italy	Revised by Viegi et al. 2003
*Anabasis articulata (*Forssk.) Moq.	Ovines	Skin diseases	Aerial part	Topic application, External	Egypt	Pieroni et al. 2006
	Caprines	Skin diseases	Aerial part	Topic application, External	Egypt	Pieroni et al. 2006
	Equines	Skin diseases	Aerial part	Topic application, External	Egypt	Pieroni et al. 2006
Apium graveolens L.	NS	Stimulate abortion	Fruits	NS	Tunisia	Revised by Viegi and Ghedira 2014
Arundo donax L.	Bovines	Diuretic and urinary antiseptic	Rhizome	Tisane, Oral (with *Smilax aspera*)	Spain	Bonet and Vallés, 2007
	Equines	Anthelmintic	Leaves	NS	Italy	Bullitta et al. 2007
	Equines	Anthelmintic	Leaves	Feed	Italy	Bullitta et al. 2018
	NS	NS	Root	Tisane, Internal	Spain	Carrió et al. 2012
	Ovines	Support for bandage	Culm	Support for bandage along with cow dung	Italy	Guarrera et al. 2015
	Bovines	Inserted in gut incision to allow fermentation products to flow slowly	Culm	Inserted in the gut incision after intoxication with *Medicago sativa*	Italy	Guarrera et al. 2015
	Equines	Stomach flatulence	Leaves	Eaten raw	Italy	Menale and Muoio 2014
	Ruminants	Alimentary tract and metabolism	NS	NS	Italy	Revised by Mayer et al. 2014
	Ruminants	Alimentary tract and metabolism	Root, bulb	NS	Italy	Revised by Mayer et al. 2014

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Atriplex halimus L.	Ovines	Digestive troubles	Seeds	Decoction of seeds mixed with honey	Algeria	Miara et al. 2019
	Bovines	Digestive troubles	Seeds	Decoction of seeds mixed with honey	Algeria	Miara et al. 2019
	Ovines	Ovarian cysts	NS	NS	Algeria	Miara et al. 2019
	Bovines	Ovarian cysts	NS	NS	Algeria	Miara et al. 2019
	Ovines	Fatten - dietary supplement	Whole plant	Dietary supplement, internal	Italy	Revised by Viegi & Ghedira 2014
	Swines	Fatten NS dietary supplement	Whole plant	Dietary supplement, internal	Italy	Revised by Viegi & Ghedira 2014
Beta vulgaris L.	NS	Intestinal antiseptic and anti-inflammatory	Leaves	Tisane , Oral (with *Parietaria officinalis* ssp. judaica and *Pisum sativum*)	Spain	Bonet & Vallés, 2007
	NS	Laxative	Leaves	Tisane, Oral	Spain	Bonet & Vallés, 2007
	NS	Postpartum coadjuvant (emollient)	Leaves	Medicinal broth, Internal	Spain	Carrió et al. 2012
	NS	Fodder	Roots	NS	Italy	Dei Cas et al. 2015
	NS	Facilitate delivery and milk production	NS	NS	Italy	Dei Cas et al. 2015
	Swines	Nutraceutical	Roots	Fresh	Albania	Pieroni et al. 2005
	NS	increasing milk secretion	Leaves	Pulp	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Abcesses and wounds	Leaves	External	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Keratoconjunctivitis	Roots	External	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Constipation	Leaves	Pulp	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Open skin wounds	Root	Powder, External	Turkey	Sinmez et al. 2018
Brassica nigra (L.) K. Koch	Bovines	LO	NS		Italy	Revised by Viegi et al. 2003
	Equines	LO	NS		Italy	Revised by Viegi et al. 2003

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Brassica nigra (L.) K. Koch	NS	Skin and wound	NS		Italy	Revised by Viegi et al. 2003
	NS	Gastrointestinal diseases	NS		Italy	Revised by Viegi et al. 2003
Brassica oleracea L.	NS	Postlabour antiseptic	Leaves	Leaves wrap the aerial part of *Mercurialis annua* L. , Direct ingestion	Spain	Bonet & Vallés, 2007
	NS	Antidiarrhoeal	Flowered aerial part	Tisane, Internal	Spain	Carrió et al. 2012
	Swines	Nutraceutical	Leaves	Fodder	Croatia	Pieroni et al. 2003
	Equines	Analgesic and anti-inflammatory (tendonitis)	Leaves	Wrapped around limb	Italy	Quave et al. 2008
	Ruminants	Gastrointestinal parasites	Leaves	Pickle, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Diarrhoea	Aerial part	Decoction, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Burns	Leaves	Mash, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Gastrointestinal parasites	Leaves	Mash, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Bruises	Leaves	Fresh, Wraps to heal bruises	Italy	Revised by Lucchetti et al. 2019
	NS	NS	NS	NS	Italy	Revised by Mayer et al. 2014
	Bovines	NS	NS	NS	Italy	Revised by Mayer et al. 2014
	NS	NS	NS	NS	Italy	Revised by Mayer et al. 2014
	NS	Products for teats and udder	Leaves	NS	Italy	Revised by Mayer et al. 2014
	NS	Musculo-skeletal system	Leaves	NS	Italy	Revised by Mayer et al. 2014
	NS	Feed or food integrator	Aerial part	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Skin and wound	NS		Italy	Revised by Viegi et al. 2003

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Brassica oleracea L.	Ovines	Skin and wound	NS		Italy	Revised by Viegi et al. 2003
	NS	LO	NS		Italy	Revised by Viegi et al. 2003
	NS	Sunburn and stroke	Leaves	Poultice, External	Turkey	Sinmez et al. 2018
Calotropis procera (Aiton) Dryand.	Camels	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	Caprines	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	Ovines	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	Camels	Scabies	Bark, Latex	Decoction	Tunisia	Revised by Viegi & Ghedira 2014
	Caprines	Scabies	Bark, Latex	Decoction	Tunisia	Revised by Viegi & Ghedira 2014
Calystegia sepium (L.) R. Br.	Rabbits	Better and healthier growth	NS	NS	Italy	Guarrera & Leporatti 2007
	Rabbits	Fodder	Aerial part	NS	Italy	Guarrera et al. 2005b
Chenopodium album L.	Swines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	NS	Fodder	Leaves	Fresh	Albania	Pieroni et al. 2005
Chenopodium ambrosioides L.	Bovines	Anthelmintic	Aerial part	NS	Spain	Revised by Mayer et al. 2014
Crithmum maritimum L.	Rabbits	Food integrator	Aerial part	NS	Italy	Cornara et al. 2009
	Rabbits	Galactogogue	Leaves	NS	Italy	Cornara et al. 2009
	Rabbits	NS	NS		Italy	Revised by Viegi et al. 2003
Cynara cardunculus L.	Ovines	Kidney problems	Leaves/Flower and Stem	Given to eat, Infusion	Algeria	Miara et al. 2019
	Ovines	Gallbladder problems	Leaves/Flower and Stem	Given to eat, Infusion	Algeria	Miara et al. 2019
	Bovines	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Cynodon dactylon (L.) Pers.	Bovines	Fodder	Whole plant	NS	Italy	Di Novella et al. 2013
	Livestock	NS	Roots	Decoction with *Malva sylvestris* leaves	Italy	Guarrera et al. 2015
	Livestock	Feed	Whole plant	NS	Italy	Idolo et al. 2010
	Swines	Feed	Aerial part	NS	Italy	Lucchetti et al. 2019
	Equines	Intestinal inflammations	Whole plant	Fodder, Internal	Italy	Pieroni et al. 2006
	NS	Increasing milk secretion	Aerial part	NS	Turkey	Revised by Erarslan & Kultur, 2019
	Equines	Feed	Aerial part	NS	Italy	Revised by Lucchetti et al. 2019
	Ruminants	Feed	Aerial part	NS	Italy	Revised by Lucchetti et al. 2019
	Ovines	Intestinal inflammations	Rhizomes	Fodder	Italy	Revised by Pieroni et al. 2004
	Ovines	Diuretic	Rhizomes	Fodder	Italy	Revised by Pieroni et al. 2004
	Equines	Food supplement	NS	NS	Italy	Revised by Viegi et al. 2003
	NS	Kidney disorders	NS	NS	Italy	Revised by Viegi et al. 2003
	NS	Gastrointestinal diseases	NS	NS	Italy	Revised by Viegi et al. 2003
	Ovines	Gastrointestinal diseases	NS	NS	Italy	Revised by Viegi et al. 2003
Daucus carota L.	Bovines	Diarrhoea	Leaves	NS	Italy	Bullitta et al. 2007
	Bovines	Gastrointestinal infection, colics, diarrhoea	Leaves	Decoction, Internal	Italy	Bullitta et al. 2018
	NS	Parasitic diseases	Fruits	Internal	Turkey	Yipel et al. 2017
	NS	Reproductive diseases	Fruits	Internal	Turkey	Yipel et al. 2017
Dittrichia viscosa (L.) Greuter	Ovines	Appetizer	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Dittrichia viscosa (L.) Greuter	Bovines	Appetizer	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Appetizer	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Appetizer	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Flatulence	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Flatulence	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Flatulence	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Flatulence	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Postpartum inflammation	Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Postpartum inflammation	Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Postpartum inflammation	Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Postpartum inflammation	Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Expectorant	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Expectorant	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Expectorant	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Expectorant	Leaves	Oral	Palestine	Ali-Shtayeh et al. 2016
	NS	Wound healing	Flowering stems	Decoction, External	Spain	Benítez et al. 2012
	NS	Intestinal anti-inflammatory	Leaves	Poultice, Topical	Spain	Bonet & Vallés, 2007
	Ovines	Stop bleeding	Aerial part	NS	Algeria	Miara et al. 2019

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Dittrichia viscosa (L.) Greuter	Ovines	Diarrhoea	Aerial part	NS	Algeria	Miara et al. 2019
	Ovines	Wounds	Aerial part	Decoction, External	Spain	Pieroni et al. 2006
	Caprines	Wounds	Aerial part	Decoction, External	Spain	Pieroni et al. 2006
	Bovines	Wounds	Aerial part	Decoction, External	Spain	Pieroni et al. 2006
	Equines	Wounds	Aerial part	Decoction, External	Spain	Pieroni et al. 2006
	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
	Equines	Anthelmintic	Aerial part	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	Ovines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Ailments affecting the locomotor apparatus	NS	Curative	Italy	Revised by Viegi et al. 2003
	Ovines	Ailments affecting the locomotor apparatus	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Anti-inflammatory to bruises	Leaves	Infusion, External	Italy	Uncini Manganelli et al. 2001
	Ovines	Anti-inflammatory to bruises	Leaves	Infusion, External	Italy	Uncini Manganelli et al. 2001
Drimia maritima (L.) Stearn	Ovines	Diarrhoea	Leaves	Decoction, Oral, add to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Diarrhoea	Leaves	Decoction, Oral, add to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Diarrhoea	Leaves	Decoction, Oral, add to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Skin diseases / Scabies	Tubers/Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Skin diseases / Scabies	Tubers/Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Drimia maritima (L.) Stearn	Caprines	Skin diseases / Scabies	Tubers/Roots	Dermal	Palestine	Ali-Shtayeh et al. 2016
	NS	Alopecia	Bulb	Direct application, External	Spain	Benítez et al. 2012
	NS	Vulnerary, antiseptic and cicatrizing	Bulb	Liniment, topical (with olive oil, *Agrimonia eupatoria*, *Sedum telephium*, *Sempervivum tectorum* and *Umbilicus rupestri*).	Spain	Bonet & Vallés, 2007
	NS	Insect reppelent	Bulb	Maceration in oil, External	Spain	Carrió et al. 2012
	Equines	Antiparasitic	Bulb	Maceration in water, External	Spain	Gonzalez et al. 2011
	Livestock	Antiparasitic (repel mice)	Bulb	Cut bulbs rubbed onto the skin (on mouse bites)	Italy	Guarrera et al. 2005a
	NS	Wounds	Leaves	Direct application	Italy	Leto et al. 2013
	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
	NS	Dermatitis	Bulb	Olive oil macerate with sulphur, External	Italy	Revised by Pieroni et al. 2004
	Caprines	'zoppina", disinfectant, vulnerary	Bulb	Crushed and blended, External	Italy	Revised by Viegi & Ghedira 2014
	NS	Wounds	Bulb	External	Italy	Revised by Viegi & Ghedira 2014
	NS	Mice reppelent	Bulb	External	Italy	Revised by Viegi & Ghedira 2014
	NS	Rodenticide	Bulb	External	Italy	Revised by Viegi & Ghedira 2014
	NS	Pruritic dermatitis	Cataphylls	External	Italy	Revised by Viegi & Ghedira 2014
	NS	Insect and mice reppelent	Whole plant	External	Italy	Revised by Viegi & Ghedira 2014
	Poultry	NS	Bulb	NS	Tunisia	Revised by Viegi & Ghedira 2014
	NS	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	NS	Antiparasitics	NS	Preventive	Italy	Revised by Viegi et al. 2003

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Echinochloa crus-galli (L.) P.Beauv.	Birds	Food supplement	NS	NS	Italy	Revised by Viegi et al. 2003
	Birds	Reconstituent	Fruit	Mixed with birdfeed	Italy	Uncini Manganelli et al. 2001
Elymus pungens (Pers.) Melderis	NS	Nervous system, psycholeptics	Fruits, Seeds, Berries	NS	Spain	Revised by Mayer et al. 2014
Elymus repens (L.) Gould	Equines	Shine coats	NS	Dietary supplement, internal	Italy	Guarrera & Leporatti 2007
	Equines	Make coat shine	Rhizomes	Added to food	Italy	Leporatti & Corradi 2000
	Equines	Skin and wound	NS	NS	Italy	Revised by Viegi et al. 2003
	Equines	Gastrointestinal diseases	NS	NS	Italy	Revised by Viegi et al. 2003
	Equines	Skin and wound	NS	NS	Italy	Revised by Viegi et al. 2003
	Bovines	NS	NS	NS	Italy	Revised by Viegi et al. 2003
	Ovines	NS	NS	NS	Italy	Revised by Viegi et al. 2003
Eucalyptus camaldulensis Dehnh.	Ovines	Fever and malaria	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Fever and malaria	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Fever and malaria	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Diarrhoea	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Diarrhoea	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Diarrhoea	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Scabies	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Scabies	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Eucalyptus camaldulensis Dehnh.	Caprines	Scabies	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Inflammation	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Inflammation	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Inflammation	Leaves	Soak or boiled, filtrated and Oral (add to drinking water)	Palestine	Ali-Shtayeh et al. 2016
	NS	Heal parasites	Leaves	Rubbed on animals	Italy	Lucchetti et al. 2019
	Ovines	Intestinal problems	Leaves	Fumigation, inhalation of steam	Algeria	Miara et al. 2019
	Bovines	Intestinal problems	Leaves	Fumigation, inhalation of steam	Algeria	Miara et al. 2019
Eucalyptus sp.	NS	Skin and wound			Italy	Revised by Viegi et al. 2003
Eucalyptus spp.	NS	Bruises	Leaves	Decoction, External	Italy	Revised by Pieroni et al. 2004
Foeniculum vulgare Mill.	Ovines	Flatulence	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Flatulence	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Flatulence	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Equines	Flatulence	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Pneumonia	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Pneumonia	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Pneumonia	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Equines	Pneumonia	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Arthritis	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Foeniculum vulgare Mill.	Bovines	Arthritis	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Arthritis	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Equines	Arthritis	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Urinary tract system	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Urinary tract system	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Urinary tract system	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	Equines	Urinary tract system	Seeds, Aerial part	Oral; Boil 250g in 1 L water, add to drinking water or to the feed	Palestine	Ali-Shtayeh et al. 2016
	NS	Wound healing	Whole plant	Decoction, Internal	Spain	Benítez et al. 2012
	Bovines	Postlabour antiseptic and anti-inflammatory	Aerial part	Tisane, Oral (with *Triticum aestivum*, *Lippia triphylla* and *Oryza sativa*)	Spain	Bonet & Vallés, 2007
	Ducks	Salutiferous	Leaves	Tisane	Spain	Bonet & Vallés, 2007
	NS	Antidiarrhoeal	Aerial part	Direct ingestion, Internal	Spain	Carrió et al. 2012
	Ovines	Food integrator	Aerial part	Mixed with shoots of *Clematis* and *Rubus*	Italy	Cornara et al. 2009
	Caprines	Fodder	Leaves	Fresh	Spain	Gonzalez et al. 2011
	Rabbits	Fodder	Aerial part	Fresh	Italy	Guarrera et al. 2006
	Bovines	Abdominal bloating	Leaves	Put in feed	Italy	Lucchetti et al. 2019
	Ovines	Galactogogue	Fruit	Fodder, Internal	Algeria	Pieroni et al. 2006
	Ovines	Galactogogue	Fruit	Fodder, Internal	Morocco	Pieroni et al. 2006
	Rabbits	Digestive	Whole plant	Decoction, Internal	Morocco	Pieroni et al. 2006
	NS	Flatulence	Aerial part	Added in fodder, Internal	Turkey	Revised by Erarslan & Kültür, 2019

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Foeniculum vulgare Mill.	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
	NS	NS	Fruits, seeds, berries	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Reproductive diseases	NS	Preventive	Italy	Revised by Viegi et al. 2003
	Bovines	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Rabbits	Food supplement		Preventive	Italy	Revised by Viegi et al. 2003
	Bovines	Ruminative	Flowers	Decoction (with *Salix alba* branches) in wine given to animals	Italy	Uncini Manganelli et al. 2001
	Bovines	Galactogogue	Fruit	Added to hay	Italy	Uncini Manganelli et al. 2001
	NS	Parasitic diseases	Fruits, Leaves, Seeds, Roots	Internal	Turkey	Yipel et al. 2017
	NS	Gastrointestinal diseases	Fruits, Leaves, Seeds, Roots	Internal	Turkey	Yipel et al. 2017
Glycyrrhiza glabra L.	NS	Antiparasitics	NS	NS	Italy	Revised by Viegi et al. 2003
	NS	Gastrointestinal diseases	Roots	Internal	Turkey	Yipel et al. 2017
	NS	Respiratory diseases	Roots	Internal	Turkey	Yipel et al. 2017
Haloxylon scoparium Pomel	Ovines	Scabies	Aerial part	Powder mixed with tobacco powder and oil and applied	Tunisia	Revised by Viegi & Ghedira 2014
Heliotropium bacciferum Forssk.	NS	Scabies	Plasters	NS	Tunisia	Revised by Viegi & Ghedira 2014
Hordeum sp.	NS	Breast lump	Aerial part, Bran	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Difficulty of birth	Aerial part, Bran	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	increasing milk secretion	Aerial part, Bran	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Pain reliever	Fruits	External	Turkey	Revised by Erarslan & Kültür, 2019

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Hordeum sp.	NS	Mastitis	Aerial part, Bran	NS	Turkey	Revised by Erarslan & Kültür, 2019
Hordeum vulgare L.	Ovines	Galactogogue	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Galactogogue	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Galactogogue	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Flatulence	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Flatulence	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Flatulence	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Constipation	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Constipation	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Constipation	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Appetizer	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Appetizer	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Appetizer	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Cleaning the uterus	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Cleaning the uterus	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Cleaning the uterus	Seeds	Oral; Boil 2kg of seeds in 6L of water add filtrate to drinking water	Palestine	Ali-Shtayeh et al. 2016
	Equines	Stomatitis, diarrhoea, intoxication	Seeds	NS	Italy	Bullitta et al. 2007
	Swines	Stomatitis, diarrhoea, intoxication	Seeds	NS	Italy	Bullitta et al. 2007

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Hordeum vulgare L.	Ovines	Stomatitis, diarrhoea, intoxication	Seeds	NS	Italy	Bullitta et al. 2007
	Bovines	Stomatitis, diarrhoea, intoxication	Seeds	NS	Italy	Bullitta et al. 2007
	Swines	Gastrointestinal infection, colics, diarrhoea	NS	Flour boiled, Internal	Italy	Bullitta et al. 2018
	Equines	Indigestion	NS	Decoction (with *Avena sativa*, *Zea mays*, *Linum usitatissimum*, water salt), Internal	Italy	Bullitta et al. 2018
	Equines	Anthelmintic	NS	Dry feed with *Santolina chamaecyparissus*	Italy	Bullitta et al. 2018
	Equines	Respiratory diseases	NS	Decoction (with *Triticum durum*), Feed	Italy	Bullitta et al. 2018
	Swines	Gastrointestinal infection, colics, diarrhoea	NS	Flour boiled, Internal	Italy	Bullitta et al. 2018
	Swines	Foot and mouth diseases (aphtha)	NS	Flour boiled, Internal	Italy	Bullitta et al. 2018
	Bovines	Gastrointestinal infection, colics, diarrhoea	NS	Flour boiled, Internal	Italy	Bullitta et al. 2018
	Bovines	Gastrointestinal infection, colics, diarrhoea	NS	Beans (*Vicia faba*) and flour boiled, Internal	Italy	Bullitta et al. 2018
	Bovines	Foot and mouth diseases (aphtha)	NS	Flour boiled, Internal	Italy	Bullitta et al. 2018
	NS	Prepare to childbirth	NS	NS	Italy	Dei Cas et al. 2015
	NS	Refreshing (combined with mash)	NS	NS	Italy	Dei Cas et al. 2015
	NS	Food	Stems deprived of kernel	NS	Italy	Dei Cas et al. 2015
	Bovines	Galactogogue	Kernels	Given with maize flour	Italy	Guarrera et al. 2005b
	Poultry	Fodder	Fruits	NS	Italy	Guarrera et al. 2015
	NS	Animal fodder	Whole plant, Seeds	NS	Turkey	Korkmaz et al. 2016
	Poultry	Kidneys	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Ovines	Kidneys	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Hordeum vulgare L.	Poultry	Cleaning the uterus after birth	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Poultry	Increase immunity	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Bovines	Kidneys	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Caprines	Kidneys	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Ovines	Diarrhoea	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Bovines	Diarrhoea	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Ovines	Cleaning the uterus after birth	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Bovines	Cleaning the uterus after birth	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Caprines	Cleaning the uterus after birth	Seeds	Heated and eaten dry / Decoction in water (energy drink)	Algeria	Miara et al. 2019
	Swines	Fodder / Nutraceutical	Seeds	NS	Italy	Pieroni et al. 2004
	Equines	Heart problems	Fruits	Boiled, vapours inhalation	Albania	Pieroni et al. 2014
	NS	Cardiotonic	NS	Boiled, given to animals	Albania	Pieroni et al. 2015
	Ovines	Gastrointestinalunfection, colics, diarrhea	NS	Flour and beans (Vicia faba) boiled , feed	Italy	Piluzza et al. 2015
	Ovines	Lack of appetite	NS	Flour with water or milk, drink	Italy	Piluzza et al. 2015
	NS	Distemper	Grain	Decoction, Internal (added to fodder)	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Strangles, emphysema	Grain	Internal	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Antiparasitic and repellents	Whole plant	NS	Italy	Revised by Mayer et al. 2014
	NS	Antiparasitic and repellents	Whole plant	NS	Italy	Revised by Mayer et al. 2014

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Hordeum vulgare L.	NS	Gastrointestinal diseases	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	NS	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
Inula sp.	Equines	Food supplement	NS	Curative	Italy	Revised by Viegi et al. 2003
	Equines	Respiratory diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
Juncus maritimus Lam.	Ovines	Urinary retention	Leaves	Infusion/Decoction	Algeria	Miara et al. 2019
	Bovines	Urinary retention	Leaves	Infusion/Decoction	Algeria	Miara et al. 2019
Lathyrus sp.	NS	Vitamin deficiency	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Difficulty of nirth	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Increasing milk secretion	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
Lolium multiflorum Lam.	Swines	Tranquilizer	Leaves	Mixed with fodder	Italy	Idolo et al. 2010
Lycium shawii Roem. & Schult.	Equines	Skin inflammations	Aerial part	Topic, External	Egypt	Pieroni et al. 2006
	Ovines	Skin inflammations	Aerial part	Topic, External	Egypt	Pieroni et al. 2006
	Bovines	Skin inflammations	Aerial part	Topic, External	Egypt	Pieroni et al. 2006
Malva arborea (L.) Webb & Berthel.	Bovines	NS	NS	Curative	Italy	Revised by Viegi et al. 2003
Medicago sp.	NS	Swelling	Whole plant	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Difficulty of nirth	Whole plant	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Retained placenta	Whole plant	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Increasing milk secretion	Whole plant	NS	Turkey	Revised by Erarslan & Kültür, 2019
Melilotus officinalis (L.) Pall.	NS	Animal fodder	Whole plant	NS	Turkey	Korkmaz et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Melilotus sp.	NS	Epidermolysis bullosa	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Reproductive diseases	NS	Preventive	Italy	Revised by Viegi et al. 2003
Peganum harmala L.	Ovines	Nervous system	Seeds	Infusion, Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Animal fertility	NS	NS	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Fever	Twigs	Vapour inhalation (Twigs are burned and animal exposed to smoke), External	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Anthelmintic	Whole plant	Powdered	Algeria	Miara et al. 2019
	Bovines	Anthelmintic	Whole plant	Powdered	Algeria	Miara et al. 2019
	Poultry	Anthelmintic	Whole plant	Powdered	Algeria	Miara et al. 2019
	Ovines	Cough	Whole plant	Mixed with fodder	Algeria	Miara et al. 2019
	Bovines	Cough	Whole plant	Mixed with fodder	Algeria	Miara et al. 2019
	Poultry	Cough	Whole plant	Mixed with fodder	Algeria	Miara et al. 2019
	Ovines	Wounds	Whole plant	Powdered	Algeria	Miara et al. 2019
	Bovines	Wounds	Whole plant	Powdered	Algeria	Miara et al. 2019
	Poultry	Wounds	Whole plant	Powdered	Algeria	Miara et al. 2019
	Ovines	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	Caprines	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	Equines	Digestive	Whole plant	Decoction, Internal	Egypt	Pieroni et al. 2006
	NS	Diarrhea	Aerial part	Decoction, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	Ovines	Appetizer	Leaves	Oral; Mix with oak and thorny burnet leaves, add to feed	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Phillyrea latifolia L.	NS	Animal blindness	NS	Internal	Turkey	Ari et al. 2018
	NS	Eye diseases (keratitis)	Leaves	Juice, External	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Eye diseases	Leaves	Crushed, Chewing, External	Turkey	Revised by Erarslan & Kültür, 2019
	Bovines	Ophtalmologicals	Leaves	NS	Turkey	Revised by Mayer et al. 2014
	NS	NS	NS	Curative	Italy	Revised by Viegi et al. 2003
Phoenix dactylifera L.	Ovines	Eye diseases including conjunctivitis	Kernels	Burned, crushed and mixed with oil, topical	Algeria	Miara et al. 2019
	Ovines	Wounds	NS	NS	Algeria	Miara et al. 2019
Phragmites australis (Cav.) Trin. ex Steud.	Equines	Stomach flatulence	Leaves	Eaten raw	Italy	Menale & Muoio 2014
	NS	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Anti-diarrhoeal	Aerial parts	Given as forage	Italy	Uncini Manganelli et al. 2001
Pistacia lentiscus L.	Ovines	Appetizer	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Appetizer	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Appetizer	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Appetizer	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Cold	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Cold	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Cold	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Cold	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Pistacia lentiscus L.	Ovines	Diarrhea	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Diarrhea	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Diarrhea	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Diarrhea	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Flatulence	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Flatulence	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Flatulence	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Flatulence	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Ovines	Increase animal fertility	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Bovines	Increase animal fertility	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Caprines	Increase animal fertility	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	Donkeys	Increase animal fertility	Aerial part	Oral	Palestine	Ali-Shtayeh et al. 2016
	NS	Distemper	Leaves	Direct application, External	Spain	Benítez et al. 2012
	Bovines	Antidermatosis (for dermatophytosis)	Leaves	Decoction, Topical (bath)	Spain	Bonet & Vallés, 2007
	Bovines	Bloat	Stems / Fruits	NS	Italy	Bullitta et al. 2007
	Livestock	Constipation	Stems / Fruits	NS	Italy	Bullitta et al. 2007
	Livestock	Scabies	Stems / Fruits	NS	Italy	Bullitta et al. 2007
	Livestock	Wound	Stems / Fruits	NS	Italy	Bullitta et al. 2007
	Bovines	Bloat	Wood	Swab after incision of vein under the belly	Italy	Bullitta et al. 2018

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Pistacia lentiscus L.	Equines	Bloat	Wood	Swab after incision of vein under the belly	Italy	Bullitta et al. 2018
	Swines	Bloat	Wood	Swab after incision of vein under the belly	Italy	Bullitta et al. 2018
	Poultry	Bloat	Wood	Swab after incision of vein under the belly	Italy	Bullitta et al. 2018
	Swines	Mange	NS	Oil scrubbed on skin	Italy	Bullitta et al. 2018
	Bovines	Mange	NS	Oil scrubbed on skin	Italy	Bullitta et al. 2018
	Bovines	Wounds	Stem / Bark	Applied on wound	Italy	Bullitta et al. 2018
	Equines	Wounds	Stem / Bark	Applied on wound	Italy	Bullitta et al. 2018
	Swines	Wounds	Stem / Bark	Applied on wound	Italy	Bullitta et al. 2018
	Poultry	Wounds	Stem / Bark	Applied on wound	Italy	Bullitta et al. 2018
	Ovines	Digestive	Leaves	Decoction, Internal	Algeria	Pieroni et al. 2006
	Bovines	Eye inflammation	Leaves, stems	Chewed by a person and expelled into eyes, External	Cyprus	Pieroni et al. 2006
	Livestock	Antiparasitic	NS	Oil scrubbed on skin	Italy	Piluzza et al. 2015
	Livestock	Bloat	Wood	Swab after incision of vein under the belly	Italy	Piluzza et al. 2015
	Ovines	Wounds	NS	Oil applied on wound	Italy	Piluzza et al. 2015
	Livestock	Wounds	Stem, bark	Powder or bark pulped applied on wound	Italy	Piluzza et al. 2015
	Livestock	Constipation	Fruits, Leaves	Decoction, Drink	Italy	Piluzza et al. 2015
	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
	NS	NS	NS	NS	Italy	Revised by Viegi et al. 2003
Plantago coronopus L.	Caprines	Birth	Whole plant	Direct application, External	Spain	Benítez et al. 2012

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Plantago coronopus L.	Poultry	Parasiticide	Leaves	Tisane, Oral	Spain	Bonet & Vallés, 2007
	Swines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
Plantago lanceolata L.	Bovines	Antidiarrhoeal	Leaves	Tisane, Oral (with *Parietaria officinalis* ssp. *judaica*, *Cynodon dactylon*, *Rubus ulmifolius* and *Malva sylvestris*)	Spain	Bonet & Vallés, 2007
	Bovines	Postlabour antiseptic	Leaves	Tisane, Oral (with *Triticum aestivum*, *Lippia triphylla* and *Tanacetum parhenium*)	Spain	Bonet & Vallés, 2007
	NS	Postpartum coadjuvant (diuretic)	Leaves	Tisane (with *Parietaria officinalis*), Internal	Spain	Carrió et al. 2012
	Livestock	Food integrator	Leaves	NS	Italy	Cornara et al. 2009
	Livestock	Fodder	Aerial part	NS	Italy	Guarrera et al. 2015
	Bovines	Insect bites	Leaves	Topic, External	Morocco	Pieroni et al. 2006
	NS	Against parasites	Leaves	Pounded (juice), External	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Abcesses	Leaves	Mash, Internal	Turkey	Revised by Erarslan & Kültür, 2019
	Poultry	Feed	Leaves	NS	Italy	Revised by Lucchetti et al. 2019
	Rabbits	Feed	Leaves	NS	Italy	Revised by Lucchetti et al. 2019
	NS	Antiparasitic and repellents	Leaves	NS	Turkey	Revised by Mayer et al. 2014
	Bovines	Alimentary tract and metabolism	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Wounds and ulcers	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Treatment of claws and hoofs	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Plantago lanceolata L.	Ovines	Reproductive diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	NS	Open skin wounds	Leaves	Poultice, External	Turkey	Sinmez et al. 2018
	NS	Dermatological diseases and wounds	Leaves	External	Turkey	Yipel et al. 2017
	NS	Respiratory diseases	Leaves	Internal	Turkey	Yipel et al. 2017
Plantago major L.	Bovines	Dug rhagades	Leaves	NS	Italy	Bullitta et al. 2007
	Bovines	Fissures (Dermatological)	NS	Boiled (with dried peritoneum of sheep), used when milking	Italy	Bullitta et al. 2018
	NS	Postpartum coadjuvant (diuretic)	Leaves	Tisane (with *Parietaria officinalis*), Internal	Spain	Carrió et al. 2012
	Livestock	Food integrator	Leaves	NS	Italy	Cornara et al. 2009
	Swines	Food (increase muscle tone decrease fat)	Leaves	Cooked	Italy	Dei Cas et al. 2015
	Equines	Feed	Plant	NS	Italy	Di Novella et al. 2013
	Ovines	Feed	Plant	NS	Italy	Di Novella et al. 2013
	Bovines	Feed	Plant	NS	Italy	Di Novella et al. 2013
	Bovines	Wounds by wolves	Leaves	Decoction	Spain	Gonzalez et al. 2017
	NS	Wounds, including with pus	Leaves	Applied on wound	Italy	Guarrera et al. 2005a
	Birds	Fodder	Seeds	Fresh/Dry	Italy	Guarrera et al. 2006
	Livestock	Haematomas	Leaves	Fresh, Applied to haematomas	Italy	Idolo et al. 2010
	Bovines	Skin infections	Leaves	Topic, External	Italy	Pieroni et al. 2006
	Ovines	Skin infections	Leaves	Topic, External	Italy	Pieroni et al. 2006
	NS	Wounds	NS	Fresh, External	Albania	Pieroni et al. 2015

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Plantago major L.	Bovines	Alimentary tract and metabolism	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Wounds and ulcers	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Bovines	Treatment of claws and hoofs	Leaves	NS	Italy	Revised by Mayer et al. 2014
	Ovines	Reproductive diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Equines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	Poultry	Respiratory diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Wound healing	Leaves	Applied on wound, alone or with olive oil	Italy	Uncini Manganelli et al. 2001
	Equines	Wound healing	Leaves	Applied on wound, alone or with olive oil	Italy	Uncini Manganelli et al. 2001
	Caprines	Wound healing	Leaves	Applied on wound, alone or with olive oil	Italy	Uncini Manganelli et al. 2001
	Birds	Wound healing	Leaves	Applied on wound, alone or with olive oil	Italy	Uncini Manganelli et al. 2001
	Ovines	Wound healing	Leaves	Applied on wound, alone or with olive oil	Italy	Uncini Manganelli et al. 2001
	Rabbits	Fodder	Leaves	NS	Italy	Vitalini et al. 2009
	NS	Wounds and insect bites	Leaves	Poultice	Italy	Vitalini et al. 2013
Plantago sp.	NS	Abcesses and wounds	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	Rabbits	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Equines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
Polygonum aviculare L.	Birds	Moult	Seed	Direct ingestion, Oral	Spain	Bonet & Vallés, 2007

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Polygonum aviculare L.	Rabbits	Fodder	NS	NS	Italy	Guarrera et al. 2015
	Rabbits	Diarrhoea	Aerial part	Fodder, Internal	Italy	Pieroni et al. 2006
	Rabbits	NS	NS	Curative	Italy	Revised by Viegi et al. 2003
Populus sp.	NS	Increasing milk secretion	Leaves	NS	Turkey	Revised by Erarslan & Kültür, 2019
	Equines	Gastrointestinal diseases	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	Rabbits	Gastrointestinal diseases	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	Equines	NS	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	Rabbits	NS	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Gastrointestinal diseases	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
	Bovines	NS	NS	Preventive, Curative	Italy	Revised by Viegi et al. 2003
Portulaca oleracea L.	Swines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Rabbits	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Poultry	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	NS	Epidermolysis bullosa	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Hairworm	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Retained placenta	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	increasing milk secretion	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Diarrhoea	Aerial part	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Gastrointestinal diseases	Leaves	Internal	Turkey	Yipel et al. 2017

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Raphanus raphanistrum L.	Bovines	Antidermatosis (for dermatophytosis)	Root	Juice by cutting roots and putting salt, Topical	Spain	Bonet & Vallés, 2007
	Equines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Equines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Rabbits	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Swines	Fodder	Whole plant	Fresh	Spain	Gonzalez et al. 2011
	Rabbits	Fodder	Whole plant	NS	Italy	Guarrera et al. 2015
	NS	Reproductive diseases	NS	Preventive	Italy	Revised by Viegi et al. 2003
Rubia tinctorum L.	Bovines	Reproductive diseases	NS	Other	Italy	Revised by Viegi et al. 2003
	NS	Gastrointestinal diseases	Roots	Internal	Turkey	Yipel et al. 2017
Rumex crispus L.	Rabbits	Food integrator	Leaves	NS	Italy	Cornara et al. 2009
	NS	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003
	Livestock	Anti-diarrhoeal	Leaves	Decoction, Oral	Italy	Uncini Manganelli et al. 2001
Rumex sp.	Birds	Food supplement	NS	NS	Italy	Revised by Viegi et al. 2003
	Birds	Food supplement	NS	NS	Italy	Revised by Viegi et al. 2003
	Equines	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Bovines	Gastrointestinal diseases	NS	Curative	Italy	Revised by Viegi et al. 2003
	Birds	Fodder / Nutraceutical	Seeds	NS	Italy	Pieroni et al. 2004
Salicornia europaea L.	NS	Feed or food integrator	Whole plant	NS	Turkey	Revised by Mayer et al. 2014

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Scirpoides holoschoenus (L.) Soják	NS	Toothache	Leaves	Direct application, Internal	Spain	Benítez et al. 2012
	NS	Cold	Leaves	Direct application, Internal	Spain	Benítez et al. 2012
	NS	Diuretic	Leaves	Direct application, Internal	Spain	Benítez et al. 2012
	NS	Cough and Cold	Branches/Stems	NS	Spain	Revised by Mayer et al. 2014
Setaria sp.	Bovines	Fodder	Aerial part	Fresh	Italy	Guarrera et al. 2006
Tamarix aphylla (L.) H.Karst.	Camels	Scabies	Gal	NS	Tunisia	Revised by Viegi & Ghedira 2014
Tamarix gallica L.	NS	NS	NS	Curative, Magic	Italy	Revised by Viegi et al. 2003
Trifolium sp.	Bovines	Fodder	Whole plant	NS	Italy	Di Novella et al. 2013
	Bovines	Nutraceutical	Aerial part	Fodder	Croatia	Pieroni et al. 2003
	Swines	Nutraceutical	Aerial part	Fodder	Croatia	Pieroni et al. 2003
	Rabbits	Nutraceutical	Aerial part	Fodder	Croatia	Pieroni et al. 2003
	NS	Galactogogue	NS	Fresh fodder	Albania	Pieroni et al. 2015
	NS	Swelling	Leaves	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Difficulty of nirth	Leaves	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Retained placenta	Leaves	NS	Turkey	Revised by Erarslan & Kültür, 2019
	NS	Increasing milk secretion	Leaves	NS	Turkey	Revised by Erarslan & Kültür, 2019
Verbena officinalis L.	NS	Wounds	Aerial part	Decoction (with *Anagallis arvensis*), External, Fresh	Spain	Akerreta et al. 2010
	Poultry	Chicken diseases	Flowered aerial part	Infusion, Internal, Fresh	Spain	Akerreta et al. 2010
	Bovines	Wounds	Aerial part	Poultice (with egg white), External, Fresh	Spain	Akerreta et al. 2010

Plant Species	Animals	EVR	Plant part (s)	Preparation and administration	Country	Reference
Verbena officinalis L.	Bovines	Problems in the udders - mastitis	Aerial part	Poultice (with egg white), External, Fresh	Spain	Akerreta et al. 2010
	NS	Wound healing	Leaves	Decoction, External	Spain	Benítez et al. 2012
	Swines	Antiseptic after castration	Leaves	Decoction, External	Spain	Pieroni et al. 2006
	NS	NS	NS	NS	Spain	Revised by Mayer et al. 2014
	NS	Skin and wound	NS	Curative	Italy	Revised by Viegi et al. 2003

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CHAPTER II

SEASONAL VARIATIONS OF THE NUTRACEUTICAL OR PHYTOTHERAPEUTIC VALUE OF SALT-TOLERANT PLANTS FOR RUMINANT ANIMALS

Research article:

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Clarifying Seasonal Biochemical Variations in Mediterranean Salt-Tolerant Plants for Sustainable Nutraceutical and Phytotherapeutic Innovations in Ruminant Health

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ABSTRACT

Climate change will increase water scarcity and soil salinization, and harm agriculture and animal production systems, especially in arid environments, such as those in Mediterranean countries. Salt-tolerant plants can offer novel solutions to support ruminant production, health and well-being, in arid areas where traditional crops fail, while mitigating climate change impacts. This work evaluated how seasonality influences biochemical assets, including crude protein, ash, total lipids, minerals, antioxidant properties and phenolics composition of nine salt-tolerant species, aiming their exploitation in ruminant production as novel nutraceutical or phytotherapeutic products. Target species included *Pistacia lentiscus* L. *Cladium mariscus* (L.) Pohl, *Inula crithmoides* L. *Helichrysum italicum* subsp. *picardii* (Boiss. & Reut.) Franco, *Calystegia soldanella* (L.) R. Br., *Medicago marina* L., *Plantago coronopus* L., *Limoniastrum monopetalum* (L.) Boiss., and *Crucianella maritima* L. Crude protein, ash, and lipids revealed notable seasonal and species-related changes. *M. marina* and *C. soldanella* had a higher protein and lower ash contents across seasons. *H. italicum* had the most consistent high content in minerals throughout seasons, coupled with antioxidant molecules. *P. lentiscus* collected in winter and *L. monopetalum* and *C. mariscus* in summer displayed increased tannin levels and high contents of anthelmintic metabolites. Findings suggest that *H. italicum*, *M. marina* and *C. soldanella* appear well-suited for nutraceutical applications, while *P. lentiscus*, *L. monopetalum*, and *C. mariscus* hold promise for development as phytotherapeutic products. This research adds to the valorization of Mediterranean salt-tolerant plants, underscoring the significance of seasonal and species-specific variability in nutrient and phytochemical composition, which display a range of opportunities for novel sustainable and tailored solutions for ruminant production systems in arid environments.

Keywords Halophytes; bioactive plants; phenolics; antioxidants; nutraceuticals; plant extracts

2.1. BACKGROUND

The Mediterranean area faces a critical challenge with expected worsening of freshwater shortages and increased soil and water salinization due to climate change (Mastrocicco & Colombani, 2021; Hassani et al., 2021). The stress on freshwater resources is intensified by the region's demographic and economic pressures, including rising demand for water in agriculture and urban areas that threaten agricultural productivity and the broader socio-economic stability of the region, highlighting the urgent need for comprehensive adaptation and mitigation strategies (Mukhopadhyay et al., 2020).

In this region, small ruminants have a substantial economic, cultural and ecological impact, mainly providing meat, milk and wool (Durmus et al., 2019). These resilient animals are well-adapted to the harsh Mediterranean conditions and take advantage of mountainous, degraded, and marginal lands (Durmus et al., 2019). However, climate change in this area will most probably challenge ruminant farming systems, by impairing animal performance and reproduction, limiting feed and forages quality, causing freshwater deficits, and altering disease's transmission patterns (Bautista-Garfias et al., 2022). Hence, novel resources that can aid to mitigate these issues urge to be discovered. For this end, bioactive plants, including salt-tolerant species, can take part in innovative integrated strategies to improve ruminant nutrition, health, and quality of its derived food products, either as feed, nutraceutical or phytotherapeutic options.

In the context of veterinary parasitology, Hoste and colleagues (2015) defined nutraceutical plants as *"a livestock feed which combines nutritional value with beneficial effects on animal health"*, whereas phytotherapeutics are plant-derived products administered to the animals for short term periods mainly for treatment purposes. Both concepts converge on the premise that plants in study are endowed with bioactive metabolites in adequate concentrations to exert beneficial effects, and polyphenols have been in the spotlight, for their numerous biological properties, including antioxidant, anti-inflammatory and anthelmintic (Hoste et al., 2015; Santos et al., 2019). The inclusion of polyphenols in the diets of small ruminants has proven to be beneficial, not only for the animals' health and productivity, but also in enhancing the quality of dairy and meat (Correddu et al., 2020; Theodorou et al., 2006; Olagaray & Bradford, 2019). Indeed, these

benefits align with the principles of the Mediterranean diet, aiming at improved health outcomes for both animals and humans.

Our research posits that salt-tolerant plants indigenous to the Mediterranean, given their resilience to aridity and saline soils, hold promise as nutraceutical plants or as sources of phytotherapeutic products targeting animal health improvement. Their robustness under Mediterranean climatic stresses may ensure a reliable resource during the semi-arid and dry seasons, which are notorious for water and feed shortages, thereby supporting more sustainable farming practices (Hasnain et al., 2023; Duarte & Caçador, 2021). Traditional utilization of various species in food and feed practices, such as *Atriplex* sp. and *Plantago* sp., underscores their suitability for animal maintenance and productivity (Petropoulos et al., 2018; Oliveira et al., 2021a; Hasnain et al., 2023; Accogli et al., 2023). Furthermore, these plants are recognized for their abundance of antioxidants, such as phenolic compounds (Lopes et al., 2016; 2023), minerals (Norman et al., 2013; 2019), and essential fatty acids (Vizzeto-Duarte et al., 2019), all contributing to improved animal health, well-being and quality of the animal products for human health.

Among different research lines proposed to validate nutraceutical plants with anthelmintic effects (Hoste et al., 2015; Torres-Fajardo et al., 2020), the assessment of the chemical composition of screened species is a key prerequisite. However, a challenge whenever exploring wild plants is that fluctuations on the biomass composition and metabolites' concentration occur, depending on plant and environmental-related factors, which consequently influence their chemical and biological properties (Azaizeh et al., 2015; Chebli et al., 2021). Hence, unveiling these changes provides valuable insights for an effective exploitation and valorization of these plants. The establishment of optimal harvesting periods, either to ensure nutritional quality or to maximize extraction of metabolites of interest (*e.g.,* polyphenols), gathers knowledge for future applications in ruminant production. Thereof, the main goal of this work was to assess the nutritional and phytochemical properties of nine selected salt-tolerant species collected in four timepoints in the Algarve, Southern Portugal, aiming its prospection as nutraceutical or therapeutical resources for ruminant animals.

2.2.METHODS

2.2.1. Plant collection and processing

This work targeted nine salt-tolerant species, namely *Pistacia lentiscus* L. (Anacardiaceae), *Cladium mariscus* (L.) Pohl (Cyperaceae), *Inula crithmoides* L. (Asteraceae), *Helichrysum italicum* subsp. *picardii* (Boiss. & Reut.) Franco (Asteraceae), *Calystegia soldanella* (L.) R. Br. (Convolvulaceae), *Medicago marina* L. (Fabaceae), *Plantago coronopus* L. (Plantaginaceae), *Limoniastrum monopetalum* (L.) Boiss. (Plumbaginaceae), and *Crucianella maritima* L. (Rubiaceae). Species were selected based on ethnoveterinary and phytochemical information available and accessibility of wild biomass. Aerial parts (including leaves, stems, flowers, and fruits, whenever present) were manually harvested in spring, summer, autumn and winter, during 2017 and 2018 (Supplementary Files, Table S1), covering four districts of Algarve, Southern Portugal (Fig. 2.1). The plant material was identified by Dr. Luísa Custódio (CCMAR), and voucher specimens kept in the XtremeBio group herbarium, at Centre of Marine Sciences (CCMAR), University of Algarve (UAlg), Portugal (Suppl. Files, Table S1). Fresh biomass was freeze-dried (Lyoalfa 15) for three days, ground using a coffee and ball miller (Retsch PM 100) until powder and kept stored in a desiccator protected from light, until extraction. The harvesting protocol was followed in accordance with the standard procedures recommended by "*Instituto da Conservação da Natureza e das Florestas (ICNF)",* the national regulatory body, and mandatory licenses for collection of wild plant material were obtained appropriately.

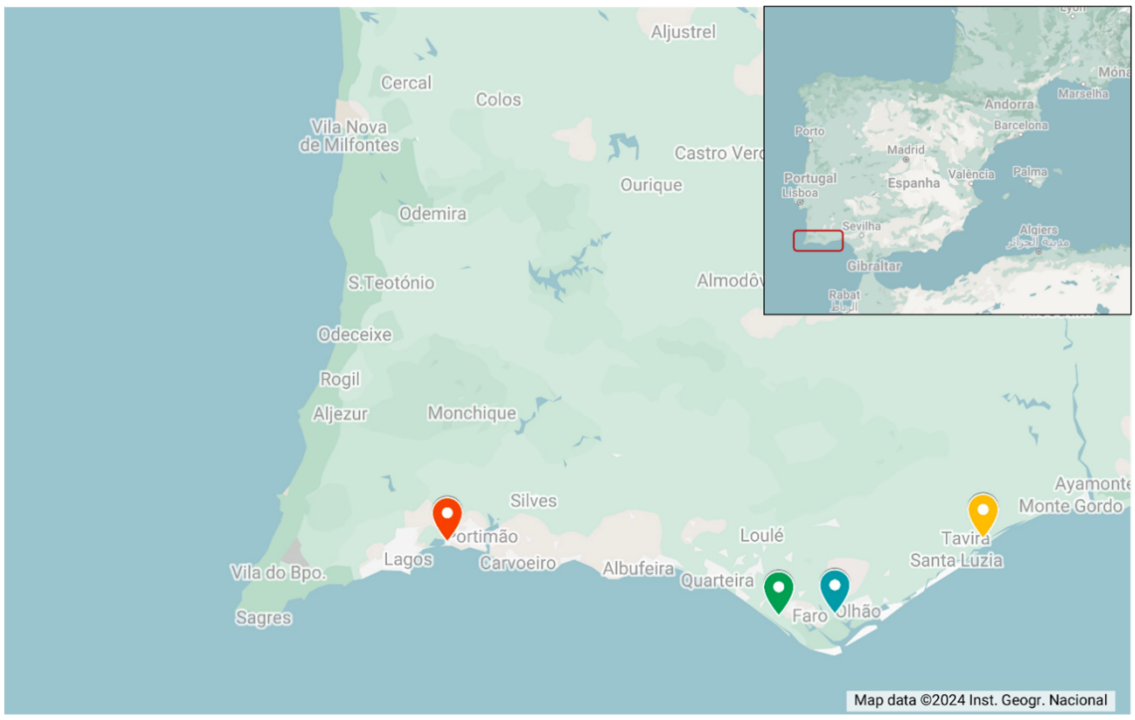

Figure 2.1 Harvesting locations of the nine salt-tolerant plants species along the Algarve coast, Southern Portugal, namely Portimão (red), Faro (green), Olhão (blue) and Tavira (yellow). Adapted from Instituto Geográfico Português ® 2024 using Google Maps.

2.2.2. Nutritional properties

Dry matter (DM) was determined by drying fresh biomass in a ventilated oven at 105 ºC, for 16 h. Ash content (A) was measured by igniting samples in a muffle furnace at 600 ºC for 2 h, following AOAC (2005) guidelines. Total nitrogen (N) was quantified using a CHN Elemental Analyzer (Vario EL III), and crude protein (CP) estimated by multiplying the N content by 6.25. Total lipids (TL) were measured using a modified Bligh and Dyer (1959) method (Bligh and Dyer, 1959). The mineral profile, including Ca, K, Na, Mg, Cu, Zn, Mn, and Fe, was determined using a microwave plasma-atomic emission spectrometer (MP-AES; Agilent 4200 MP-AES, Agilent Victoria, Australia), as detailed elsewhere (Oliveira et al., 2021b). Total carbohydrates (CHO) were estimated as the residue upon subtraction of CP, TL and A values whilst gross energy (GE; Mcal kg^{-1}) was given by the equation (GE = (CPx0.056)+(TLx0.094)+[(100-CP-TL-A)x0.042], as described by Weiss and Tebbe, 2019.

2.2.3. Preparation of the extracts

Dried ground biomass was extracted with an 80% water-based acetone solution (1:40, *w/v*) at 20-25 ºC, for 16 h under constant stirring. The extracts were filtered, concentrated

in a rotary evaporator under reduced pressure and temperature (40 ºC), dissolved in dimethyl sulfoxide (DMSO) and stored at -20 ºC until use.

2.2.4. Total phenolic (TPC), flavonoid (TFC) and condensed tannins contents (CTC) of the extracts

Total phenolics, flavonoids, and condensed tannins were assessed as fully described in (Oliveira et al., 2021b). In summary, total phenolic content (TPC) was estimated using the Folin-Ciocalteau (F-C) method, total flavonoid content (TFC) by the aluminum chloride ($AlCl_3$) method, and condensed tannin content (CTC) was evaluated using the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA-HCl) colorimetric protocol, adapted to 96-well microplates. Calibration curves were prepared using gallic acid, quercetin, and catechin, respectively. The concentrations of TPC, TFC, and CTC were expressed as gallic acid equivalents (GAE), quercetin equivalents (QE), and catechin equivalents (CE), respectively, in mg per gram of extract dry weight (mg eq. $extract^{-1}$, DW).

2.2.5. Phenolics profiling by high performance liquid chromatography-diode array (HPLC-DAD)

The extracts (10 mg mL^{-1}) in a mixture of 90% ultrapure water and 10% MeOH were analyzed by HPLC-DAD (Agilent 1100 Series LC system, Germany), constituted by the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostatic column compartment (G1316A) and the diode array detector (G1315B). The data acquisition and instrumental control were performed by the software LC3D ChemStation (version Rev.A.10.02[1757], Agilent Technologies). Analyses were performed on a Mediterranea sea18 column, 15 × 0.21 cm, 5 µm particle size (Teknokroma, Spain). The mobile phase consists on a mixture of methanol (solvent A) and 2.5% acetic acid aqueous solution with the following gradient: 0-5 min: 10 % A, 5-10 min: 10-30 % A, 10-40 min: 30-90 % A, 40-45 min: 90 % A, 45-55 min: 90-10 % A and 55-60 min: 10 % A. The flow used was 0.35 mL min^{-1} with an injection volume of 40 µL and a draw speed of 200 µL min^{-1}. The detector was set at 210, 280 (used for quantification), 320 and 350 nm. For identification, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV-visible

spectral reference data. The levels of the different compounds were extrapolated from calibration standard curves. Commercial standards of gallic acid, 3-4-dihdroxybenzoic acid, neochlorogenic acid, *p*-hidroxybenzoic acid, catechin hydrate, 4-hydroxybenzaldehyde, 3-hydroxybenzoic acid, vannilic acid, chlorogenic acid, 4-*O*-caffeoylquinic acid, caffeic acid, syringic acid, epigallocatechin gallate, epicatechin, ourateacatechin, umbeliferone, coumaric acid, taxifolin, coumarin, ferulic acid, salicyclic acid, naringenin-7-glucoside, luteolin-7-*O*-glucoside, rosmarinic acid, rutin, ellagic acid, cinnamic acid, quercetin, morin, flavone and chrysin were prepared in methanol (1 mg L^{-1}) and diluted with ultrapure water.

2.2.6. *In vitro* antioxidant properties

The radical scavenging activity (RSA) on $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ free radicals was assessed as described in detail in (Oliveira et al., 2021b). Extracts were tested at various concentrations (from 10 to 0.078 mg mL^{-1}) to determine the half inhibitory concentrations (IC_{50} values). Absorbance was measured in a multiplate spectrophotometer reader (Biotek Synergy 4). For all assays, except the ferric reduction antioxidant power (FRAP), results were expressed as a percentage of inhibition compared to the negative control (DMSO) and as IC_{50} values (mg mL^{-1}) when possible; the FRAP assay results were compared to a positive control (ascorbic acid). Butylated hydroxytoluene (BHT; 1 mg mL^{-1}) was used as positive control for DPPH and ABTS whilst ethylenediamine tetraacetic acid (EDTA; 1 mg mL^{-1}) was used for the metal chelation assays.

2.2.7. Statistical analysis

All experiments were performed, at least, in duplicate, with results expressed as mean ± standard error of the mean (SEM). Antioxidant data is presented as the concentration required to achieve 50% of antioxidant activity (IC_{50} value), inferred by sigmoidal fitting of the transformed data (GraphPad Prism Software v.5.0). Seasonal effects on phenolics contents and antioxidant activities were analyzed using IBM SPSS Statistics v.20.0 software with analysis of variance (ANOVA), and significance between means was evaluated (p = 0.05; assuming parametricity of the data). Spearmen's correlations between mineral contents were performed using the XLSTAT statistical add-on for Microsoft Excel.

2.3.RESULTS

2.3.1. Nutritional properties

Results for crude protein, ash and total lipids are summarized in Fig. 2.2, and fully detailed on Supplementary Files, Table S2. Crude protein levels in the target species ranged between 39.4 to 189.4 g kg^{-1} DM, across seasons. In general, protein levels were frequently low in the dry periods (summer/autumn), most species barely meeting the minimum level required for animal maintenance (70 g kg^{-1} DM) (Hasnain et al., 2023). In contrast, during spring, most species had adequate levels of protein (87.3 - 132.7 g kg^{-1} DM), exceptions including *P. lentiscus* and *C. maritima* while in winter, *I. crithmoides*, *L. monopetalum*, *P. coronopus*, *M. marina* and *C. soldanella* samples exceeded this threshold (91.4-189.4 g kg^{-1} DM). Overall, the highest protein values were observed in *M. marina* during winter/spring (132.7 - 139.4 g kg^{-1} DM) and, exceptionally, in *C. soldanella* in winter (189.4 g kg^{-1} DM). Total lipid content across samples ranged from 34.4 to 250.1 g kg^{-1} DM, showing variation among species. *L. monopetalum* recorded the lowest levels (34.4 - 44.1 g kg^{-1}DM), followed by *C. mariscus* (48.9-53.3 g kg^{-1} DM) and *M. marina* (45.9-55.1 g kg^{-1} DM). Conversely, the highest fat contents were registered in *C. soldanella* (141.9-250.1 g kg^{-1} DM), showing levels declining in dry seasons, and *H. italicum* (90.9-142.7 g kg^{-1} DM), which peaked in autumn/winter. Ash content showed a broad range, from 49.3 to 326.6 g kg^{-1} DM, depending on species and season. While *H. italicum*, *P. lentiscus*, *C. mariscus*, and *M. marina* exhibited adequate ash levels, *L. monopetalum* and *I. crithmoides* stood out for their remarkably high ash content (148.3–326.6 g kg^{-1} DM), accompanied by lower GE values (3.3.-3.8 Mcal g^{-1}; Suppl. Files, Table S2). As illustrated by Weiss & Tebbe (2019), GE is deeply dependent on the ash, lipids and protein concentrations, and thereof, it decreases with substantial increases in ash. In agreement, highest GE values were recorded for *C. soldanella* samples, particularly in spring (GE = 5.2 Mcal kg^{-1}), and *H. italicum* (GE = 4.5-4.7 Mcal kg^{-1}), following the trend of higher lipid contents.

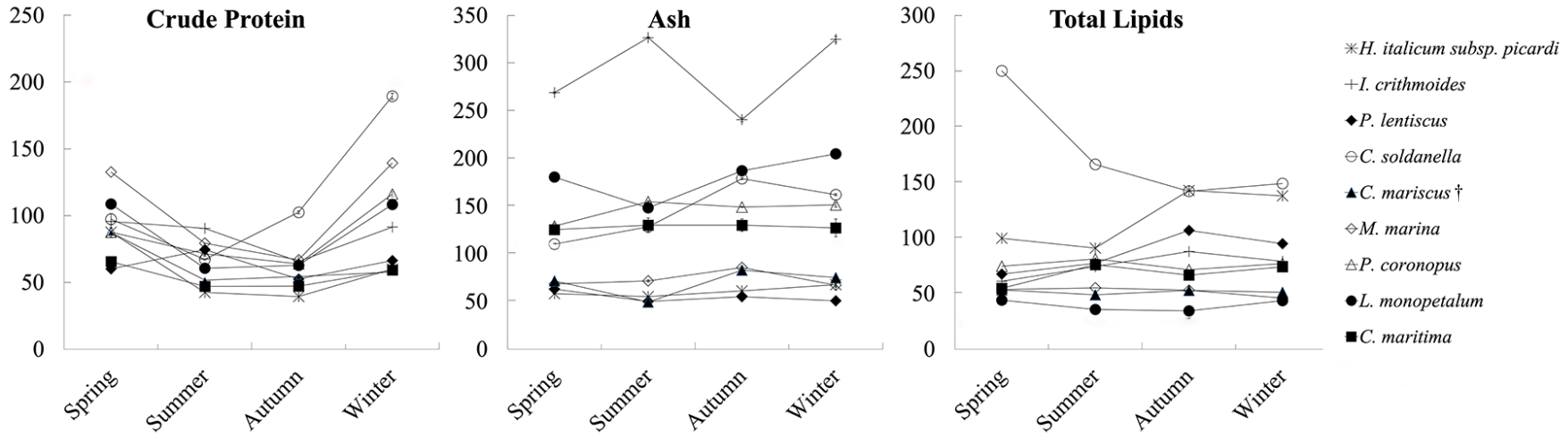

Figure 2.2 Seasonal variations of the crude protein, ash and total lipid contents (g kg^{-1} DM) of salt-tolerant species biomass. † Data published in Oliveira et al., 2021b.

The high ash values were largely attributed to elevated sodium (Na) and magnesium (Mg) levels, as evidenced by significant positive correlations between ash and these elements (ash/Na, 0.842; ash/Mg, 0.802; Na/Mg, 0.765; $p < 0.001$; Spearmen's correlations). Macromineral analysis revealed high levels throughout the year for most samples, with some exceptions: *C. mariscus* had reduced potassium (K) and Mg levels, while *M. marina* exhibited lower K content during autumn (Fig. 2.3). Regarding trace minerals, except for *C. mariscus* in winter, all samples surpassed Fe dietary recommendations (40 mg kg^{-1} DM), remaining within maximum tolerable levels (MTL). Similarly, the Zn content of most samples was adequate, apart of *L. monopetalum* in summer, whilst *H. italicum* was the only species with high levels of copper (Cu) and manganese (Mn) for all seasons. In fact, *H. italicum* stood out since its macro- and trace mineral profiles were consistently greater throughout seasons for every element tested. *I. crithmoides, C. mariscus, P. coronopus* and *C. maritima* exhibited Cu contents adequate for animal maintenance, whereas *C. soldanella* had increased levels in autumn/winter. Following *H. italicum, C. maritima* and *C. mariscus* showed high levels of Mn in the four sampled seasons, along with *C. soldanella* collected in summer and autumn. Significant positive correlations between Mn, Cu, and Zn were also noted (Mn/Cu: 0.682; Mn/Zn: 0.594; Zn/Cu: 0.476; $p < 0.001$, Spearman's correlations), highlighting potential interactions that may influence their nutritional contributions.

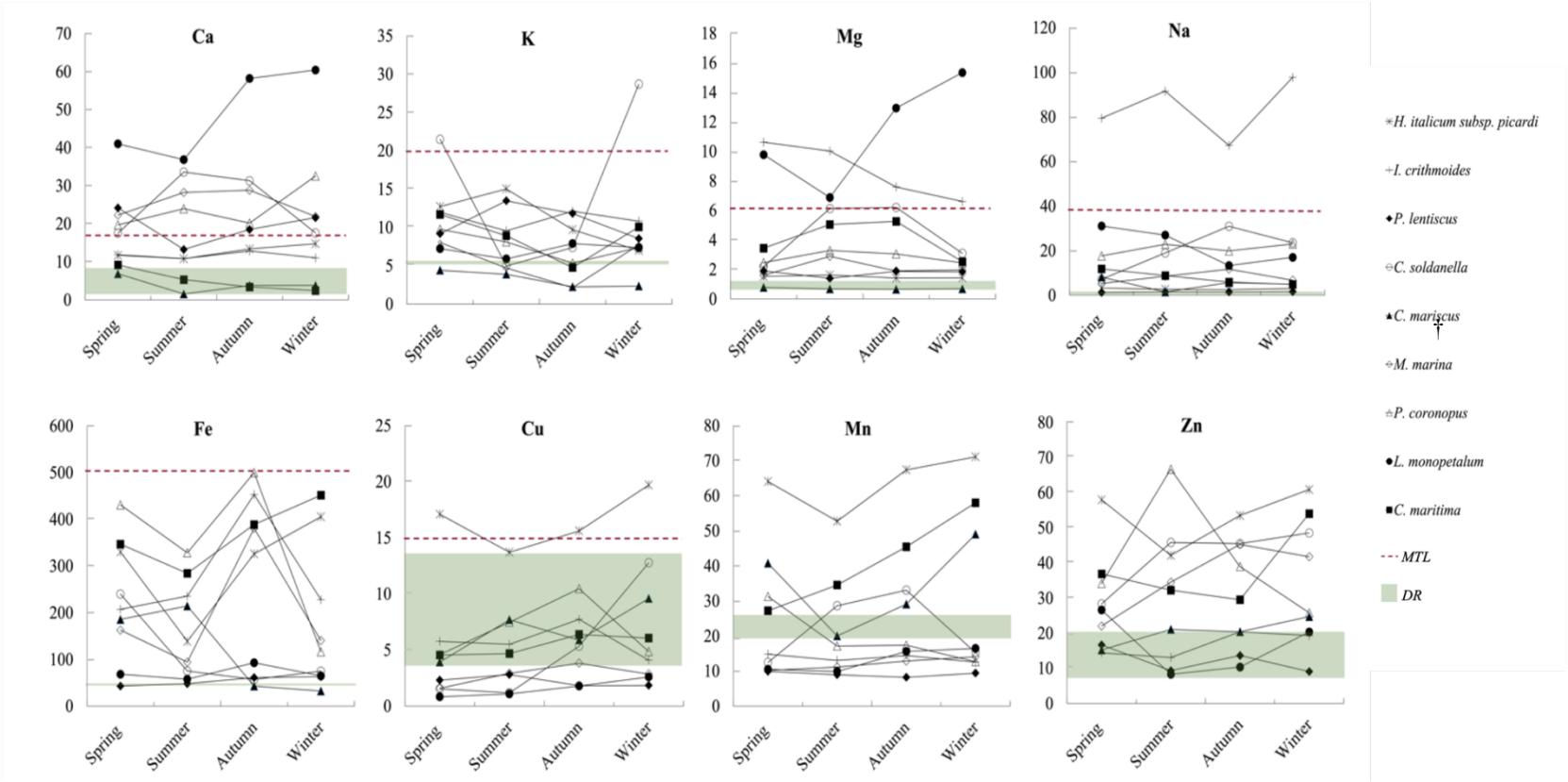

Figure 2.3 Seasonal profile of macrominerals (Ca, K, Mg and Na; g kg^{-1} DM) and trace minerals (Fe, Cu, Mn, Zn; mg kg^{-1} DM) evaluated in the dry biomass of salt-tolerant species. Dietary requirement (**DR**) limits are represented as green bars: the lower limit recalls to minimum dietary concentration required for animal maintenance while the upper limit to reproduction, lactation, and rapidly growing animals (Freer et al., 2007). Maximum tolerable levels (**MTL**) are represented as a dashed red line (NRC, 2005), and above this line, dietary concentrations will have negative impacts on animal health. † Data published in Oliveira et al., 2021b. **Ca**, Calcium, **K**, Potassium, **Mg**, Magnesium, **Na**, Sodium, **Fe**, Iron, **Cu**, Copper, **Mn**, Manganese, **Zn**, Zinc. Dietary requirements (**DR): Ca**, 1.4-7 g kg^{-1} DM; **K**, 5 g kg^{-1} DM; **Mg**, 0.9-1.2 g kg^{-1} DM; **Na**, 0.7-1.0 g kg^{-1} DM; **Fe**, 40 mg kg^{-1}; Cu, 4-14 mg kg^{-1} DM; Mn, 20-25 mg kg^{-1} DM; **Zn**, 9-20 mg kg^{-1} DM. Maximum tolerable levels (**MTL;** sheep): **Ca**, 15 g kg^{-1} DM; **K**, 20 g kg^{-1} DM; **Mg**, 6 g kg^{-1} DM; **Na**, 40 g kg^{-1} DM; **Fe**, 500 mg kg^{-1} DM; **Cu**, 15 g kg^{-1} DM; **Mn**, 2000 mg kg^{-1} DM; **Zn**, 300 mg kg^{-1} DM.

2.3.2. Phytochemical profiling

The accumulation of total phenolics (Fig. 2.4-A), total flavonoids (Fig. 2.4-B) and condensed tannins (Fig. 2.5) was assessed. Except for *M. marina* and *I. crithmoides*, a high TPC was noted for all samples (>20 mg g^{-1} DW; Fig. 2.4-A; Kähkönen et al., 1999; Rodrigues et al., 2015), ranging from 23 up to 230 mg GA eq. g^{-1} extract DW. In a descendent order, TPC values were estimated as follows: *P. lentiscus* > *P. coronopus* > *L. monopetalum* > *C. mariscus* > *H. italicum* > *C. soldanella* > *C. maritima*. Seasonal patterns varied widely among salt-tolerant species. *H. italicum* and *I. crithmoides* had higher TPC in autumn (105.6 and 27.2 mg GA eq. g^{-1} extract DW, respectively), while *L. monopetalum* and *C. soldanella* phenolics content increased in spring (144.8 and 73.2 mg GA eq. g^{-1} extract DW). *P. lentiscus* and *C. maritima* samples reached the lowest values in summer (195.2 and 19.3 mg GA eq. g^{-1} extract DW), while no differences were observed for other seasons. *P. coronopus* had higher contents in winter (160 mg GA eq. g^{-1} extract DW) while *M. marina* exhibited a marked increase in spring/summer (13.3-14.2 mg GA eq. g^{-1} extract DW). *C. mariscus* best results were achieved in the dry periods (summer/autumn; 104.3 - 112.3 mg GA eq. g^{-1} extract DW) in contrast to spring (86. mg GA eq. g^{-1} extract DW). Total flavonoids levels ranged between 4.8 and 45.4 mg QE eq. g^{-1} extract DW. *I. crithmoides* showed consistently the lowest TFC values (4.8 - 13.3 mg QE eq. g^{-1} extract DW), while *C. soldanella* and *H. italicum* had the highest levels, particularly in spring/summer samples (42 - 45.2 and 35.8 - 45.4 mg QE eq. g^{-1} extract DW, respectively). In general, the highest amounts of flavonoids were recorded in spring/summer samples, except for *I. crithmoides* and *P. lentiscus*, which had increased contents in autumn (13.3 mg QE eq. g^{-1} extract DW) and winter (28.9 mg QE eq. g^{-1} extract DW), respectively.

Condensed tannins were only detected in three species: *P. lentiscus* > *L. monopetalum* > *C. mariscus* (Fig. 2.5). Comparably to TPC, seasonal variations did not follow a trend, showing species-specific traits. An increased accumulation of these metabolites in *P. lentiscus* was detected in winter, *L. monopetalum* peaked in summer and *C. mariscus* was consistently a rich source of these metabolites in summer, autumn and winter.

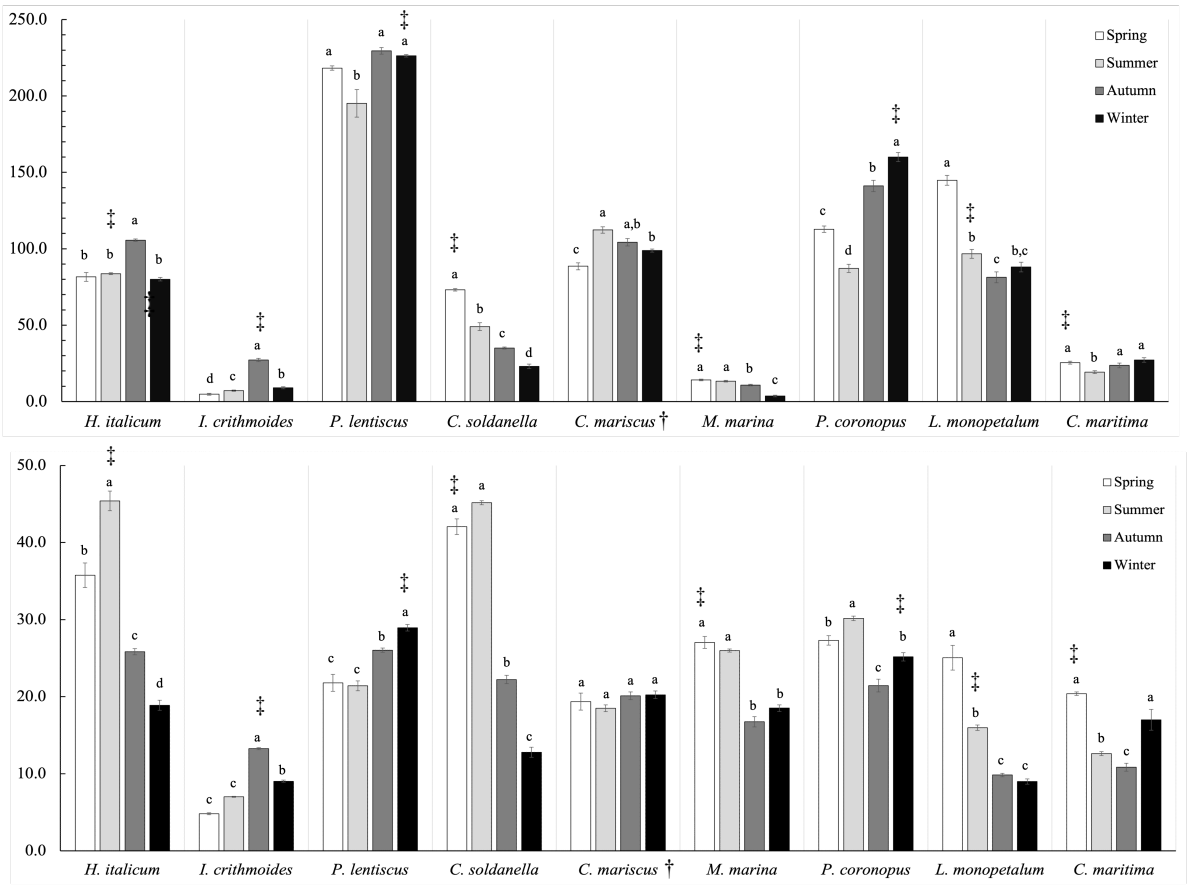

Figure 2.4 Seasonal variations of the total phenolic (TPC; **A**) and total flavonoids (TFC; **B**) contents of 80% acetone extracts of salt-tolerant species. Results are expressed as mg gallic acid equivalents g^{-1} extract DW (TPC) and mg quercetin equivalents g^{-1} extract DW (TFC). Different letters refer to significant differences for each species across seasons per species ($p < 0.05$). † Data published in Oliveira et al., 2021b; ‡, Data published in Oliveira et al., 2021c.

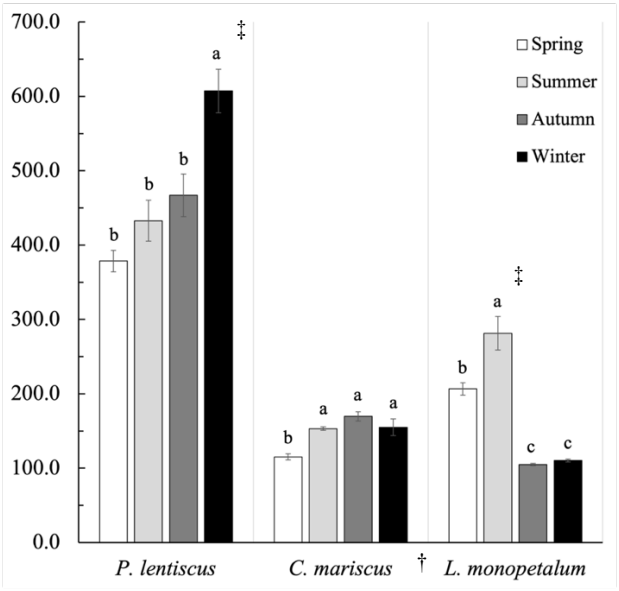

Figure 2.5 Seasonal dynamics of the condensed tannin contents (CTC) of 80% acetone extracts obtained from three salt-tolerant plant species. Results are expressed as mg catechin equivalents g^{-1} extract DW. Different letters refer to significant differences for each species between seasons ($p < 0.05$). † Data published in Oliveira et al., 2021b; ‡, data published in Oliveira et al., 2021c.

2.3.3. Antioxidant properties

Regarding antioxidant properties, and in alignment with similar studies (Lopes et al., 2016; Pereira et al., 2024), only five species were selected for analysis as they exhibited IC_{50} values below 1 mg mL^{-1} in at least two antioxidant assays (Table 2.1). Notably, none of the samples demonstrated iron-chelating capacity, and only *P. lentiscus* and *L. monopetalum* consistently presented IC_{50} values below 1 mg mL^{-1} for copper chelation. In the latter, seasonal variations were negligible for *P. lentiscus*, while *L. monopetalum* showed consistent activity during spring, summer, and winter (IC_{50} = 0.58–0.66 mg mL^{-1}). In the DPPH assay, *C. mariscus* and *P. lentiscus* displayed stable antiradical activity across all seasons within each respective species ($p < 0.05$). Conversely, the other three species exhibited significant seasonal variability: *H. italicum* samples were mostly active during summer and autumn (IC_{50} = 0.27–0.29 mg mL^{-1}); *L. monopetalum* showed a decrease in activity only during autumn (IC_{50} = 0.18 mg mL^{-1}); and *P. coronopus* consistently demonstrated lower IC_{50} values in spring, winter, and autumn (IC_{50} = 0.17–0.25 mg mL^{-1}; Table 2.1).

Table 2.1 Antioxidant capacity of 80% acetone extracts prepared from five salt-tolerant plant species collected across seasons. Results are expressed as the concentration that inhibits 50% of the radical (IC_{50} values), in mg mL^{-1}. Different letters correspond to significant differences for each season in each assay ($p < 0.05$).

Species	Season	CCA	DPPH	FRAP	ABTS
H. italicum picardii	Spring	>1	0.43 ± 0.01^{c}	0.74 ± 0.11^{b}	0.63 ± 0.13^{a,b}
	Summer	>1	0.29 ± 0.01^{a,b}	0.32 ± 0.02^{a}	0.30 ± 0.02^{a}
	Autumn	>1	0.27 ± 0.00^{a}	0.45 ± 0.04^{a}	0.30 ± 0.06^{a}
	Winter	>1	0.33 ± 0.01^{b}	0.40 ± 0.03^{a}	0.86 ± 0.14^{b}
P. lentiscus	Spring	0.18 ± 0.02^{a}	0.03 ± 0.01^{a}	0.07 ± 0.00^{a,b}	0.03 ± 0.00^{a}
	Summer	0.13 ± 0.02^{a}	0.04 ± 0.01^{a}	0.07 ± 0.00^{a,b}	0.04 ± 0.00^{a,b}
	Autumn	0.18 ± 0.02^{a}	0.04 ± 0.00^{a}	0.09 ± 0.01^{b}	0.04 ± 0.01^{b}
	Winter	0.17 ± 0.01^{a}	0.03 ± 0.00^{a}	0.05 ± 0.00^{a}	0.03 ± 0.00^{a}
C. mariscus †	Spring	>1	0.30 ± 0.00^{a}	0.25 ± 0.02^{a}	0.29 ± 0.03^{b}
	Summer	>1	0.24 ± 0.02^{a}	0.21 ± 0.03^{a}	0.12 ± 0.01^{a}
	Autumn	>1	0.25 ± 0.01^{a}	0.18 ± 0.05^{a}	0.20 ± 0.02^{a,b}
	Winter	>1	0.26 ± 0.03^{a}	0.27 ± 0.01^{a}	0.23 ± 0.03^{b}
P. coronopus	Spring	>1	0.25 ± 0.02^{a,b}	0.23 ± 0.01^{a}	0.47 ± 0.06^{a}
	Summer	>1	0.30 ± 0.03^{b}	0.29 ± 0.01^{a}	0.55 ± 0.13^{a}
	Autumn	>1	0.20 ± 0.03^{a}	0.32 ± 0.05^{a}	0.40 ± 0.10^{a}
	Winter	>1	0.17 ± 0.02^{a}	0.20 ± 0.01^{a}	0.33 ± 0.04^{a}
L. monopetalum	Spring	0.65 ± 0.03^{a}	0.12 ± 0.01^{a}	0.09 ± 0.01^{a}	0.11 ± 0.02^{a,b}
	Summer	0.58 ± 0.03^{a}	0.12 ± 0.01^{a}	0.13 ± 0.01^{a,b}	0.06 ± 0.01^{a}
	Autumn	0.92 ± 0.11^{b}	0.18 ± 0.00^{b}	0.18 ± 0.03^{b}	0.15 ± 0.02^{a,b}
	Winter	0.66 ± 0.04^{a,b}	0.14 ± 0.01^{a}	0.11 ± 0.01^{a,b}	0.16 ± 0.03^{b}

† Data published in Oliveira et al., 2021b. EDTA was used as positive control for CCA (IC_{50} = 0.08±0.01 mg mL^{-1}), ascorbid acid for FRAP (IC_{50} = 0.03±0.01 mg mL^{-1}) and BHT for DPPH (IC_{50} = 0.14±0.00 mg mL^{-1}) and ABTS (IC_{50} = 0.11±0.01 mg mL^{-1}).

Overall, the most promising combined results for phenolic content and antioxidant activity were observed in *P. lentiscus* and *P. coronopus* samples collected during winter, as well as in *L. monopetalum*, *C. mariscus*, and *H. italicum* harvested during summer. Based on these findings, the phenolic profiles of these samples were further explored through HPLC-DAD analysis (Fig. 2.6; Table 2.2).

2.3.4. Individual phenolic composition

The individual chromatograms revealed notable differences, reflecting the chemical complexity of the extracts coupled with species-specific variations. In total, 19 compounds were identified in *P. lentiscus* (winter; 138.91 mg phenolics g extract DW^{-1}),

23 in *H. italicum* (summer; 27.91 mg phenolics g extract DW^{-1}), 14 in *C. mariscus* (summer; 9.22 mg phenolics g extract DW^{-1}) [31], 22 in *P. coronopus* (winter; 23.03 mg phenolics g extract DW^{-1}), and 21 in *L. monopetalum* (summer; 25.43 mg phenolics g extract DW^{-1}).

The predominant metabolites in *C. mariscus* (summer) were phenolic acids, including chlorogenic acid (**10**; 2.96 mg g extract DW^{-1}), ferulic acid (**21**; 1.38 mg g extract DW^{-1}), and salicylic acid (**22**; 1.64 mg g extract DW^{-1}; Oliveira et al., 2021b). *P. lentiscus* stood out as a rich source of ourateacatechin (**16**; 34.72 mg g extract DW^{-1}), gentisic acid (4; 33.80 mg g extract DW^{-1}), epicatechin (**15**; 25.59 mg g extract DW^{-1}), gallic acid (**1**; 13.81 mg g extract DW^{-1}), and vanillic acid (**9**; 12.62 mg g extract DW^{-1}), together contributing 120.54 mg out of 138.91 mg phenolics g extract DW^{-1}. In *H. italicum* (summer) sample, prominent phenolics included chlorogenic acid (**10**; 9.12 mg g extract DW^{-1}), neochlorogenic acid (**3**; 2.52 mg g extract DW^{-1}), epicatechin (**15**; 2.38 mg g extract DW^{-1}), ourateacatechin (**16**; 1.76 mg g extract DW^{-1}), salicylic acid (**22**; 2.59 mg g extract DW^{-1}), and luteolin-7-*O*-glucoside (**24**; 3.58 mg g extract DW^{-1}), accounting for 21.95 mg of the total 27.91 mg phenolics g extract DW^{-1}. In *P. coronopus* (winter), dominant compounds were gentisic acid (**4**; 5.53 mg g extract DW^{-1}), salicylic acid (**22**; 5.36 mg g extract DW^{-1}), and luteolin-7-O-glucoside (**24**; 5.36 mg g extract DW^{-1}), which together made up 16.25 mg of the total 23.03 mg phenolics g extract DW^{-1}. The *L. monopetalum* (summer) extract was rich in caffeic acid (**12**; 6.79 mg g extract DW^{-1}), gallic acid (**1**; 3.79 mg g extract DW^{-1}), gentisic acid (**4**; 3.79 mg g extract DW^{-1}), chlorogenic acid (**10**; 2.12 mg g extract DW^{-1}), 3-hydroxybenzoic acid (**8**; 1.24 mg g extract DW^{-1}), and 4-O-caffeoylquinic acid (**11**; 1.76 mg g extract DW^{-1}).

Interestingly, certain compounds displayed species-specific occurrences. For instance, 4-*O*-caffeoylquinic acid (**11**) was unique to *L. monopetalum*, while morin (**30**) and chrysin (**32**) were exclusively detected in *H. italicum* samples. Conversely, luteolin-7-O-glucoside (**24**), epicatechin (**15**), caffeic acid (**12**), and gallic acid (**1**) were common across all species, albeit in varying concentrations. Although several unresolved peaks remain, these findings highlight the distinct phenolic profiles of each species, offering opportunities for their targeted exploitation.

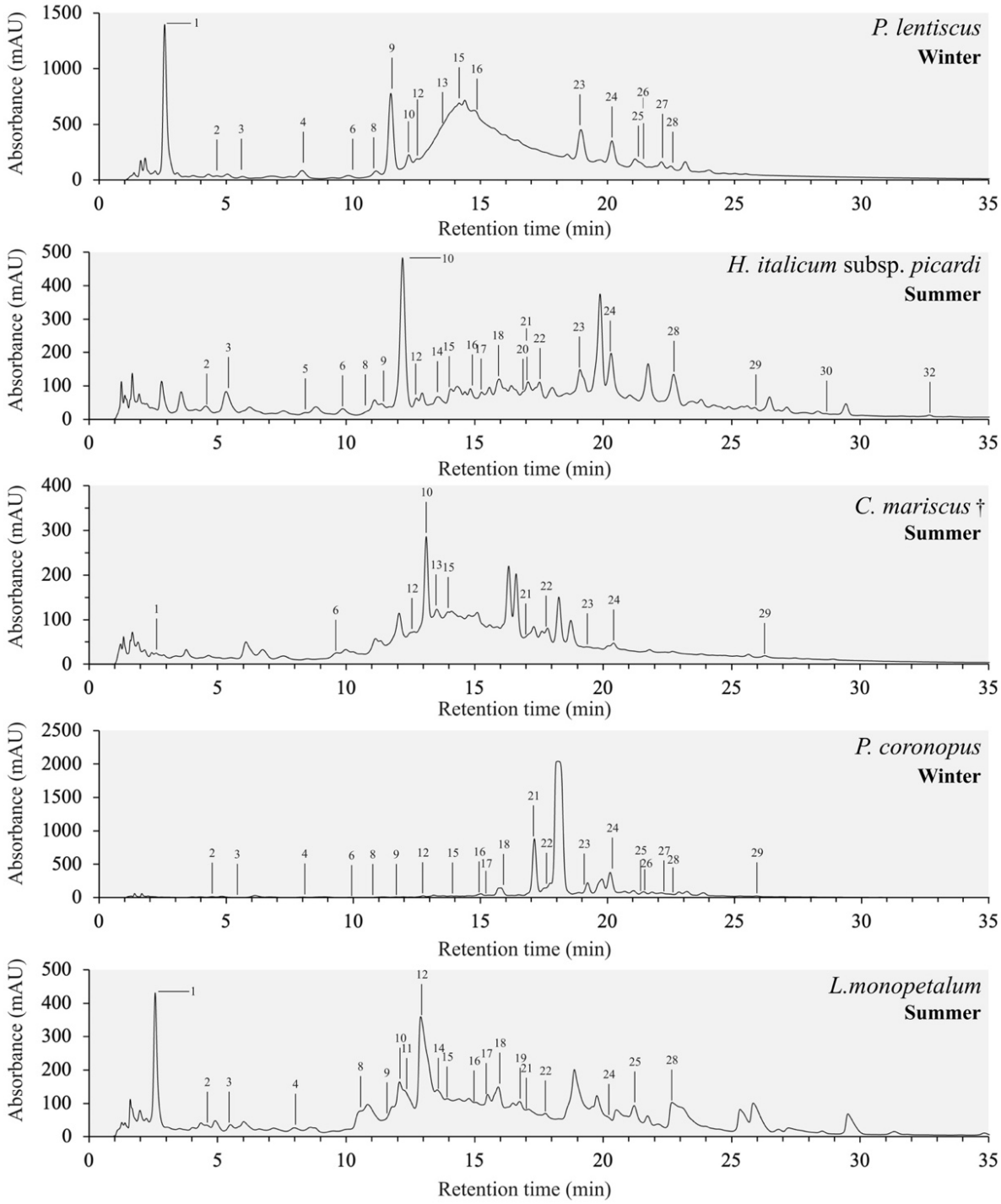

Figure 2.6 Peak chromatograms of *P. lentiscus, H. italicum picardii, C. mariscus*, *P. coronopus* and *L. monopetalum* selected 80% acetone extracts, using HPLC-DAD. Peaks are numbered according to its retention time (RT). † data published in Oliveira et al., 2021b.

Table 2.2 Identification and quantification of individual phenolic metabolites (mg g extract^{-1}) on *P. lentiscus, H. italicum picardii, C. mariscus*, *P. coronopus* and *L. monopetalum* selected 80% acetone extracts, using HPLC-DAD detection. Compounds are numbered according to its retention time (RT). † Data published in Oliveira et al., 2021b, and retention times may differ for some compounds.

RT (min)	No.	Compound	*P. lentiscus* Winter	*H. italicum* Summer	*C. mariscus* Summer †	*P. coronopus* Winter	*L. monopetalum* Summer
2.6	**1**	Gallic acid	13.81	<0.01	0.01	<0.01	3.79
4.6	**2**	3.4-Dihydroxybenzoic acid	0.22	0.36	n.d.	0.33	0.27
5.4	**3**	Neochlorogenic acid	0.39	2.52	n.d.	0.07	0.52
8.1	**4**	Gentisic acid	33.80			5.53	3.79
8.4	**5**	*p*-Hidroxybenzoic acid		0.17	< 0.01		
9.9	**6**	Catechin hydrate	2.43	1.92	0.77	0.12	
10.0	**7**	4-Hydroxybenzaldehyde			< 0.01		
10.7	**8**	3-Hydroxybenzoic acid	0.84	0.19	n.d.	0.14	1.24
11.6	**9**	Vanillic acid	12.62	0.14		0.05	0.41
12.2	**10**	Chlorogenic acid	1.29	9.12	2.96		2.12
12.3	**11**	4-*O*-Caffeoylquinic acid			n.d.		1.76
12.6	**12**	Caffeic acid	0.74	0.89	0.73	0.76	6.79
13.5	**13**	Syringic acid	2.62		0.35		
13.6	**14**	Epigallocatechin gallate		0.05		<0.01	0.72
13.9	**15**	Epicatechin	25.59	2.38	0.88	0.51	0.57
14.9	**16**	Ourateacatechin	34.72	1.76	n.d.	0.41	0.95
15.2	**17**	Umbelliferone		0.15	n.d.	0.41	0.05
15.9	**18**	Coumaric acid	<0.01	0.16	< 0.01	1.19	0.40
16.8	**19**	Taxifolin			n.d.		0.36
16.9	**20**	Coumarin		0.01	n.d.		
17.0	**21**	Ferulic acid		0.51	1.38	0.81	0.06

17.6	**22**	Salicylic acid		2.59	1.64	5.36	0.48
19.1	**23**	Naringenin-7-glucoside	2.78	0.82	0.01	1.18	
20.3	**24**	Luteolin-7-*O*-glucoside	5.47	3.58	0.46	5.36	0.23
21.3	**25**	Rosmarinic acid	0.60			0.12	0.58
21.4	**26**	Rutin	0.54			0.09	
22.2	**27**	Ellagic acid	0.24			0.26	<0.01
22.6	**28**	Cinnamic acid	0.21	0.38	n.d.	0.26	0.34
25.9	**29**	Quercetin		0.06	0.03	0.07	
28.8	**30**	Morin		0.03	n.d.		
31.7	**31**	Flavone					
32.6	**32**	Chrysin		0.12	n.d.		
		∑ Phenolics	**138.91**	**27.91**	**9.22**	**23.03**	**25.43**

2.4. DISCUSSION

The prospection of plants intended for nutraceutical uses must firstly focus on gathering nutritional evidence (Hoste et al., 2015; Torres-Fajardo et al., 2020). Crude protein values are often a decisive factor to establish the nutritional quality of botanical resources, since it influences biomass intake and rumen function, and a sharp decline occurs when the level is below 70 g kg^{-1} DM (Hasnain et al., 2023). In this work, protein levels were frequently low in the dry periods (summer/autumn), most species barely reaching the 70 g kg^{-1} mark, consistent with other non-traditional salt-tolerant fodder species of the Mediterranean basin (Zahran & El-Amier, 2013; El-Amier et al., 2022). Whilst most samples sustain animal maintenance, higher values would be required for growing or lactating stages of animal production systems (140-180 g kg^{-1} DM; Hasnain et al., 2023). Considering the importance of protein on animal growth, reproduction, and lactation (SCA, 2007), the low values reported for dry periods (average for summer = 65 g kg^{-1} DM and autumn = 61.7 g kg^{-1} DM) may constrain its use in times when there is a manifest need for alternatives. Furthermore, the anticipated climate shifts in the Mediterranean region, characterized by prolonged droughts, higher temperatures, and shorter rainfall periods, will likely extend the duration of the dry season (Giorgi & Bi, 2005; Alo et al., 2017). It is crucial to acknowledge that while local flora is adapted to such conditions, these environmental changes may still influence their growth cycles and, by extension, their nutritional profiles during what are currently considered wetter seasons. Despite these challenges, the scarcity of plants that can thrive in these increasingly arid conditions underscores the invaluable role of salt-tolerant species, either as feed complement or as source of specific nutrients (El Shaer, 2010).

A constraint often mentioned as a limiting factor to salt-tolerant species feeding value is its high ash content (Attia-Ismail, 2008; El Shaer, 2010; Hasnain et al., 2023). Although it has no energy value, minerals are essential for ruminant production, yet many pastures fail to provide it in enough amounts to sustain high levels of animal productivity year-round (Masters et al., 2019). While all species demonstrated adequate macromineral levels for maintenance purposes, most proved to be rich sources of microminerals throughout the year, capable of supporting various levels of production (Fig. 2.3). Copper (Cu), Mn, zinc (Zn), selenium (Se), and sulfur (S) are particularly valuable due to their

roles in antioxidant defense mechanisms (Norman et al., 2019), and strong positive correlations between these elements were identified. Trace minerals like Cu, Mn, Se, and Zn, alongside bioactive molecules such as phenolics, offer added value by mitigating oxidative stress-related disorders commonly experienced during ruminant production, such as reproduction, lactation, heat stress, and gastrointestinal parasitic infections (Celi, 2011). These benefits extend to enhancing the quality of meat and dairy products (Castillo et al., 2013; Norman et al., 2019; Olagaray and Bradford, 2019), positioning these salt-tolerant species as functional resources for improved nutrition and production sustainability. As a main point, this study underscores the opportunity to exploit these plants as dietary supplements or nutraceutical products, addressing mineral imbalances across seasons.

Although carbohydrates and gross energy were estimated (Suppl. Files, Table S2), a detailed assessment of fiber, palatability, *in vitro*/*in vivo* digestibility, and impact on animal production of these species should be addressed in future work, to complement the data herein gathered. It is worth mentioning that animal intake also depends on the assortment of plants being consumed: animals may refuse to consume high-protein plants and accept low-protein and tannin-rich forages to counteract the negative effects of the excess protein in the first ones (Ventura-Cordero et al., 2017; Castañeda-Ramirez et al., 2018). *H. italicum* included in mixed feeding strategies, along with two other shrub species such as *Juniperus phoenicea* L. 1753 and *Juniperus oxycedrus* L. 1753, was widely consumed by small ruminants, despite their low protein content and high terpene levels (Rogosic et al., 2006a). In agreement, small ruminant's intake of Mediterranean shrubs with high tannin contents increased when offered in multiple mixed feeding schemes (Rogosic et al., 2006b).

Despite being perceived as anti-nutritional factors (depending on dose), phenolics are also molecules of antioxidant (Lopes et al., 2016) and anthelmintic interest (Hoste et al., 2015; Santos et al., 2019) for ruminant animals. The synthesis of these bioactive molecules is often triggered by environmental factors, leading to fluctuations on pharmacological effects. Thereof, the seasonal dynamics assessment of the phenolic profile and antioxidant properties herein presented targets a bidirectional approach: it complements the nutraceutical evaluation of these plants, aiming their use as bioactive plants, while prospecting extracts as phytotherapeutic products.

Natural products with antioxidant properties offer significant health benefits for small ruminants by reducing oxidative stress, which can improve immune function, support overall health, and enhance productivity. Herein, IC_{50} values obtained for summer extracts of *C. mariscus*, *L. monopetalum* and *P. lentiscus* were equal or lower than those reported by others testing 70% acetone extracts from summer harvested biomass (Lopes et al., 2016). Overall, *P. lentiscus* and *P. coronopus* samples collected in winter, along with *L. monopetalum*, *C. mariscus*, and *H. italicum* harvested in summer, exhibited the most promising combined results on phenolic content and antioxidant activity. Individual chromatograms of these samples obtained through an HPLC-DAD analysis were quite distinct, following the chemical complexity of the extracts and species-related differences. *P. lentiscus* was rich in ourateacatechin (**16**), gentisic acid (**4**), epicatechin (**15**)**,** gallic acid (**1**) and vanillic acid (**9**), consistent with the findings comprehensively reviewed by Sehaki et al. (2023) from different works, who summarized a diverse range of phenolics in this species, encompassing various classes such as flavonoids, flavonols, flavanols, flavones, flavonoid glycosides, myricetin derivatives, anthocyanins, catechins, and phenolic acids along with their derivatives. *H. italicum* (summer) sample had higher contents of chlorogenic acid (**10**), neochlorogenic acid (**3**), epicatechin (**15**), ourateacatechin (**16**), salicylic acid (**22**) and luteolin-7-*O*-glucoside (**24**). Using LC-MS, Pereira and colleagues (2017a) detected mainly quinic and chlorogenic acids, syringic acid, caffeic acid, astragalin, hyperin and oleanolic acids in decoctions and infusions of aerial organs and flowers of *H. italicum picardii*, collected in the same location, during June 2013. Samples from the species *P. coronopus* (winter) contained mainly gentisic acid (**4**), salicylic acid (**22**) and luteolin-7-*O*-glucoside (**24**;). Compounds **2-4**, **8, 14**, **16-17**, **23**, **26-28** were not detected previously in the work of Pereira and colleagues (2017b), while in reverse verbascoside, apigenin and the aglycone luteolin were not herein identified (Table 2.2). As verbascoside is a main metabolite of *P. coronopus*, it is plausible that the unresolved peak at 18min corresponds to this compound. *L. monopetalum* (summer) accumulated caffeic acid (**12**), gallic acid (**1**), gentisic acid (**4**), chlorogenic acid (**10**), 3-hydroxybenzoic acid (**8**), and 4-*O*-caffeoylquinic acid (**11**). Working with the same species, Trabelsi et al., (2012) quantified eleven phenolics (gallic, p-hydroxybenzoic, chlorogenic, syringic, vanillic, ferulic, and trans-cinnamic acids,

quercetin, apigenin, amentoflavone and flavone) in leaf extracts after acidic hydrolysis from Tunisian plants collected in May 2006.

Besides antioxidant credit, several metabolites herein identified and quantified have been priorly examined for their anthelmintic properties, on different life stages (*e.g.*, L3 larvae, eggs) of gastrointestinal nematodes (GIN), many displaying significant activity, such as gallic (**1**), caffeic (**12**), chlorogenic (**10**), cinnamic, vanillic (**9**) and ferulic (**21**) acids, epicatechin (**15**), catechin (**6**) ourateacatechin (**16**), taxifolin (**19**), rutin (**26**), quercetin (**29**), morin (**30**) and chrysin (**32**; Brunet and Hoste, 2006; Klongsiriwet et al., 2015; Mancilla-Montelongo et al., 2019; Sprengel Lima et al., 2021). This opens the venue for these plants to be prospected as novel sustainable options for fighting GIN infections in ruminant production systems, either as nutraceutical plants or phytotherapeuticals.

Structure-activity studies on a wide variety of natural phenolics reveal that the antioxidant and anthelmintic effects of individual metabolites depend on the phenolic class, rely on structural differences and that synergistic and antagonistic interactions occur between metabolites (Barrau et al., 2005; Spiegel et al., 2020; Šamec et al., 2021; Santos et al., 2021), which adds to the complexity of their biological effects. In the end, the multitude of interactions coupled with the unresolved peaks in the chromatograms hampers our knowledge on up to which extent each one is contributing to the bioactive traits.

2.5. CONCLUSIONS

This study demonstrates that optimal harvesting periods for salt-tolerant plants vary depending on the target species and looked-for biotechnological applications, whether for maximizing specific nutrients, extracting valuable metabolites, or achieving a combination of both. *M. marina* and *C. soldanella* exhibit a more balanced nutritional profile across seasons, characterized by higher protein and lower ash contents, making them promising candidates for feed or nutraceutical applications; *I. crithmoides*, *L. monopetalum* and *P. coronopus*, may serve as viable alternatives, particularly in seasons when ash content is lower; *H. italicum* is notable for its high mineral and antioxidant content, indicating its potential as a feed supplement or nutraceutical resource; and *P. lentiscus* (winter), *L. monopetalum* and *C. mariscus* (summer) stand out due to their

elevated tannin levels and abundance of identified anthelmintic metabolites, presenting opportunities for their exploration as phytotherapeutic products or sources of bioactive compounds.

Overall, this work enhances our understanding of how seasonal variations influence the suitability of salt-tolerant plant biomass for diverse applications in ruminant production systems. It underscores the value of these species as year-round resources, maximizing their potential for feed, nutraceuticals, and phytotherapeutics. By integrating seasonal dynamics into the decision-making process, this study provides a framework for optimizing the utilization of salt-tolerant plants to support sustainable ruminant production systems.

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SUPPLEMENTARY FILES

Table S1 Plant harvesting details, including voucher number, collection dates and organs and climacteric data. Meteorological data is expressed as monthly mean values (IPMA). Seasons: Sp, spring, Su, summer, Au, Autumn, Wi, winter; Aerial organs: L leaves, S stems, FR fruits, FL flowers, I inflorescences.

Species / Family / Voucher no.	Location	Season	Date	Organs	x̄ Min. Temp. (ºC)	x̄ Max. Temp. (ºC)	x̄ Rainfall (mm)
Helichrysum italicum subsp. *picardii* (Asteraceae) (voucher number: XBH32)	Tavira 37° 07′ 51.8″ N, 7° 36′ 37.6″ W	Spring	Apr/2017	L/S	14	24	25
		Summer	Jul/2017	L/S/FL	18	30	1
		Autumn	Oct/2017	L/S/FL	14	26	25
		Winter	Jan/2018	L/S	6	16	50
Inula crithmoides (Asteraceae) (voucher number: XBH04)	Olhão 37° 01′ 11.7″ N, 7° 53′ 04.8″ W	Spring	Apr/2017	L/S	14	24	25
		Summer	Jul/2017	L/S	18	30	1
		Autumn	Oct/2017	L/S/FL	16	26	25
		Winter	Jan/2018	L/S	6	16	50
Pistacia lentiscus (Anacardiaceae) (voucher number: XBH06)	Portimão 37° 07′ 34.7″ N, 8° 36′ 02.3″ W	Spring	Apr/2017	L/S	10	24	10
		Summer	Jul/2017	L/S	14	30	1
		Autumn	Oct/2017	L/S/FR	12	26	10
		Winter	Jan/2018	L/S/FR	2	16	100
Cladium mariscus (Cyperaceae) (voucher number: XBH03)	Faro 37° 01′ 03.3″ N, 7° 59′ 18.1″ W	Spring	Apr/2017	L	14	22	25
		Summer	Jul/2017	L/I	18	30	1
		Autumn	Oct/2017	L/I	16	26	25
		Winter	Jan/2018	L	6	16	50
Calystegia soldanella (Convolvulaceae) (voucher number: XBH07)	Portimão 37° 07′ 23.1″ N,	Spring	Apr/2018	L/S/FL	10	20	10
		Summer	Jul/2018	L/S	12	26	1

Species / Family / Voucher no.	Location	Season	Date	Organs	x̄ Min. Temp. (ºC)	x̄ Max. Temp. (ºC)	x̄ Rainfall (mm)
	8° 36′ 10.7″ W	Autumn	Oct/2017	L/S	12	26	10
		Winter	Jan/2018	L/S/FL	2	16	100
Medicago marina (Fabaceae) (voucher number: XBH41)	Portimão 37° 07′ 23.1″ N, 8° 36′ 10.7″ W	Spring	Apr/2018	L/S/FL	10	20	10
		Summer	Jul/2018	L/S/FR	12	26	1
		Autumn	Oct/2017	L/S	12	26	10
		Winter	Jan/2018	L/S	2	16	100
Plantago coronopus (Plantaginaceae) (voucher number: XBH02)	Olhão 37° 01′ 32.8″ N, 7° 53′ 04.4″ W	Spring	Apr/2018	L/S/FL	12	20	100
		Summer	Jul/2018	L/S/FL	16	26	1
		Autumn	Oct/2017	L/S/FL	16	26	25
		Winter	Jan/2018	L	6	16	50
Limoniastrum monopetalum (Plumbaginaceae) (voucher number: XBH05)	Portimão 37° 07′ 34.7″ N, 8° 36′ 02.3″ W	Spring	Apr/2017	L/S	10	24	10
		Summer	Jul/2017	L/S/FL	14	30	1
		Autumn	Oct/2017	L/S	12	26	10
		Winter	Jan/2018	L/S	2	16	100
Crucianella maritima (Rubiaceae) (voucher number: XBH40)	Portimão 37° 07′ 23.2″ N, 8° 36′ 12.3″ W	Spring	Apr/2017	L/S	10	24	10
		Summer	Jul/2017	L/S/FL	14	30	1
		Autumn	Oct/2017	L/S	12	26	10
		Winter	Jan/2018	L/S	2	16	100

Table S2 Seasonal assessments of the chemical composition and mineral content of aerial parts of the nine selected salt-tolerant plants. Nutritional parameters (DM, CP, A, TL and CHO) and macrominerals (Ca, K, Mg and Na) are expressed in g kg^{-1} DM, trace minerals (Fe, Cu, Mn, Zn) in mg kg^{-1} DM and GE as Mcal kg^{-1}.

Species	Season	Nutritional parameters					Energy	Mineral composition							
		DM	CP	A	TL	CHO	GE	Ca	K	Mg	Na	Cu	Fe	Mn	Zn
Helichrysum italicum subsp. *picardii* (Asteraceae)	Spring	314.6	87.6	58.1	99.6	754.6	4.6	11.7	12.7	1.5	3.2	17.1	330.2	64.0	58.0
	Summer	458.6	42.5	55.1	90.9	811.5	4.5	10.8	15.0	1.6	2.7	13.7	138.8	52.7	42.2
	Autumn	549.4	39.4	61.2	142.7	756.7	4.7	13.4	9.7	1.4	2.4	15.6	325.9	67.4	53.5
	Winter	496.3	60.0	67.6	137.8	734.7	4.7	14.7	7.0	1.4	3.0	19.7	405.2	71.0	60.9
Inula crithmoides (Asteraceae)	Spring	97	95.7	269.0	61.1	574.2	3.5	11.8	12.0	10.6	79.4	5.8	207.3	14.8	14.3
	Summer	161.8	90.5	326.6	74.2	508.7	3.3	10.8	9.5	10.0	91.5	5.5	235.5	13.0	14.0
	Autumn	124.6	65.6	240.6	87.7	606.0	3.7	12.9	12.1	7.6	67.2	7.7	452.9	14.4	20.3
	Winter	104.3	91.4	324.9	79.0	504.7	3.4	11.0	10.8	6.6	97.7	4.1	227.8	12.6	20.5
Pistacia lentiscus (Anacardiaceae)	Spring	484.5	60.1	63.0	67.5	809.4	4.4	24.2	9.2	1.9	1.2	2.3	43.0	9.9	16.7
	Summer	494.6	74.5	50.2	77.3	798.1	4.5	13.2	13.5	1.4	1.5	2.8	48.0	8.9	9.3
	Autumn	479.9	52.5	55.1	106.7	785.7	4.6	18.5	11.8	1.9	1.5	1.8	61.0	8.2	5.3
	Winter	504.3	66.4	50.8	94.9	787.9	4.6	21.6	8.5	1.8	1.6	1.8	62.9	9.4	9.1
Calystegia soldanela (Convolvulaceae)	Spring	121.4	97.3	110.2	250.1	542.5	5.2	31.3	7.4	6.2	30.9	5.4	57.0	33.1	45.5
	Summer	84.3	67.2	128.4	166.0	638.4	4.6	17.6	28.7	3.1	23.5	12.8	74.8	15.3	48.5
	Autumn	163.3	102.5	178.9	141.9	576.7	4.3	17.5	21.5	2.2	7.1	1.5	239.8	12.4	28.3
	Winter	224.6	189.4	161.9	148.8	499.8	4.6	33.6	5.0	6.1	19.0	1.2	75.1	28.6	45.8
Cladium mariscus (Cyperaceae)	Spring	449.1	87.3	71.7	57.5	783.6	4.3	6.8	4.4	0.8	8.1	6.0	123.7	40.7	15.2
	Summer	585.5	51.8	49.3	43.2	855.7	4.3	1.6	3.9	0.7	1.4	6.3	214.3	20.0	21.1
	Autumn	559.1	54.5	82.9	55.4	807.3	4.2	3.8	2.3	0.7	5.7	5.7	43.0	29.0	20.4
	Winter	469.4	57.7	75.1	53.7	813.6	4.2	3.8	2.3	0.7	4.9	20.5	32.0	49.0	24.7

Species	Season	Nutritional parameters					Energy	Mineral composition							
		DM	CP	A	TL	CHO	GE	Ca	K	Mg	Na	Cu	Fe	Mn	Zn
Medicago marina (Fabaceae)	Spring	376.6	132.7	68.5	53.8	745.1	4.4	28.8	2.1	1.9	11.7	3.8	380.6	12.9	45.3
	Summer	307	79.4	71.7	55.1	793.9	4.3	21.9	7.7	2.0	6.7	2.8	140.3	14.0	41.7
	Autumn	271.8	66.9	85.7	53.0	794.5	4.2	22.3	8.0	1.6	5.3	1.6	162.7	10.2	22.0
	Winter	400.2	139.4	67.1	45.9	747.7	4.4	28.2	4.7	2.9	8.5	2.9	94.3	11.1	34.5
Plantago coronopus (Plantaginaceae)	Spring	251.6	87.6	129.1	74.6	708.7	4.2	20.2	5.3	3.1	19.9	10.5	499.9	17.3	38.9
	Summer	127.6	71.5	154.7	81.0	692.8	4.1	32.5	7.4	2.4	23.0	4.9	116.3	12.6	25.8
	Autumn	152.5	63.9	149.1	71.5	715.5	4.0	19.7	9.8	2.5	17.6	4.6	430.8	31.3	34.1
	Winter	280.9	116.1	151.3	76.9	655.6	4.1	23.9	8.1	3.3	22.8	7.5	328.2	17.1	66.7
Limoniastrum monopetalum (Plumbaginaceae)	Spring	291.3	108.7	180.5	44.1	666.7	3.8	41.0	7.2	9.8	31.0	0.8	68.2	10.5	26.7
	Summer	374.2	60.6	148.3	35.7	755.4	3.8	36.8	5.9	6.9	27.0	1.1	57.6	9.9	10.5
	Autumn	495.5	62.8	187.1	34.4	715.8	3.7	58.2	7.9	12.9	13.3	1.8	92.9	15.6	9.0
	Winter	322.8	108.3	204.8	43.7	643.2	3.7	60.4	7.4	15.3	17.0	2.6	64.7	16.4	20.3
Crucianella maritima (Rubiaceae)	Spring	174.8	65.6	125.3	54.7	754.4	4.1	9.2	11.7	3.4	11.8	4.5	346.9	27.2	36.9
	Summer	473.3	47.0	130.0	75.9	747.0	4.1	3.4	8.9	5.0	8.8	4.7	284.4	34.5	32.3
	Autumn	573.9	47.2	130.0	66.6	756.2	4.1	3.4	4.8	5.3	5.9	6.4	388.7	45.4	29.5
	Winter	359.9	59.2	127.1	74.0	739.7	4.1	2.4	10.1	2.5	4.8	6.1	451.5	58.0	54.0

DM, Dry matter; **CP**, Crude protein; **TL**, Total lipids; **CHO,** total carbohydrates; **GE**, gross energy; **Ca**, Calcium; **K**, Potassium; **Mg**, Magnesium; **Na**, Sodium; **Cu**, Copper; **Fe,** Iron; **Mn,** Manganese; **Zn,** Zinc.

CHAPTER III

DISCLOSING THE BIOACTIVE METABOLITES INVOLVED IN THE *IN VITRO* ANTHELMINTIC EFFECTS OF SALT-TOLERANT PLANTS

Research article:

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Disclosing the bioactive metabolites involved in the *in vitro* anthelmintic effects of salt-tolerant plants through a combined approach using PVPP and HPLC-ESI-MS^n

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ABSTRACT

Strategies to reduce dependence on synthetic drugs for the treatment of gastrointestinal nematodes (GIN) infections in ruminants include the search for novel anthelmintic scaffolds on plants, yet salt-tolerant plants remain overlooked. This study aims to evaluate the *in vitro* anthelmintic properties of selected salt-tolerant plants against GIN, and identify the potential bioactive secondary metabolites involved. For that purpose, 80% acetone/water extracts were prepared from dried biomass of aerial organs of nine salt-tolerant plant species and tested against *Haemonchus contortus* and *Trichostrongylus colubriformis* by the Larval Exsheathment Inhibition Assay (LEIA) and Egg Hatching Inhibition Assay (EHIA). *Pistacia lentiscus, Limoniatrum monopetalum, Cladium mariscus* and *Helichrysum italicum picardii* were the most active in both GIN and life stages. To investigate the role of polyphenols in the anthelmintic activity, four selected extracts were treated with polyvinylpolypyrrolidone (PVPP), and non-treated and treated samples were further characterized by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n). While polyphenols seem responsible for the EHIA properties, they are partially accountable to LEIA results. Several phenolics involved in the anthelmintic effects were identified and discussed. In sum, these species are rich sources of anthelmintic compounds and, therefore, are of major interest for nutraceutical and/or phytotherapeutic applications against GIN in ruminants.

Keywords: halophytes; *Haemonchus* sp.; *Trichostrongylus* sp.; nutraceuticals; phytotherapeutic products

3.1. INTRODUCTION

Ruminants' production represents an important agricultural sector in the Mediterranean basin, accounting for approximately 267 million heads of cattle, sheep and goats in 2019, according to FAOSTAT. The global prevalence of gastrointestinal nematodes (GIN) represents a challenge to ruminants' production in outdoors systems of production since infections have a significant impact on animal health and welfare, performance and quality of animal products (*e.g.,* milk), with consequent economic losses and without control, being causes of significant morbidity and mortality (Hoste et al., 2010; Mavrot et al., 2015). *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp, and *Nematodirus* spp. are the major relevant GIN species in Europe (Charlier et al., 2018). For the last 70 years, the control of GIN has relied mostly on the repeated administration of single or combinations of synthetic anthelmintic drugs, belonging to different 'broad-spectrum" anthelmintic such as (i) benzimidazoles, (ii) levamisole, morantel, (iii) macrocyclic lactones, and (iv) monepantel (Besier et al., 2016). However, resistances to the different drug families are nowadays reported worldwide against different GIN species in different ruminants' species (Rose Vineer et al., 2020). There is also an increasing number of references on GIN populations presenting multi-resistance to several anthelmintic families. These results have encouraged the pursuit of novel sustainable and alternatives for a more integrated control with reduced reliance on synthetic anthelmintic treatments.

Plants and their bioactive products stand out as one of these non-chemical sustainable approaches to counteract GIN infections (Hoste et al., 2015). The anthelmintic properties of legume forage with containing polyphenols, including bird foot trefoil (*Lotus corniculatus* L.), big trefoil (*L. pedunculatus* Cav.), sulla (*Hedysarum coronarium* L.; Niezen et al., 1998a,b), and sainfoin (*Onybrichis viciifolia* Scop.; Paolini et al., 2004), inspired further research on similar effects among other botanical groups that could be used as nutraceuticals, but also that may represent potential sources of novel phytotherapeutic products and active principles of pharmacological interest (Hoste et al., 2015). So far, several plant extracts, fractions, and individual compounds have been studied for their potential anthelmintic properties (Zangueu et al., 2018; Santos et al., 2019). The main bioactive compounds of interest for anthelmintic activity are

polyphenols, particularly condensed tannins and flavonoids, but others such as terpenoids, proteinases, and saponins have also been described (Santos et al., 2019).

A wide number of extremophile plants, including salt-tolerant species, occur in the Mediterranean area (Le Houérou,1994). They are adapted to harsh environmental conditions, such as high sunlight exposure, UV radiation, drought, and salinity. One of these plants' evolutionary strategies to cope with such constraints includes the production and accumulation of secondary metabolites, particularly flavonoids and tannins (Di Ferdinando et al., 2014). Additionally, former investigations reveal that many species exhibit relevant bioactive properties, like antioxidant, anti-inflammatory, and enzyme inhibitory activities (Lopes et al., 2016) with diverse applications, including in veterinary medicine. Moreover, some species have ethnoveterinary uses (Oliveira et al., 2021), for example, *Pistacia lentiscus* L., which is used as antiparasitic, for the treatment of bloat, constipation, and dermatological ailments in sheep and goats (Piluzza et al., 2015). Nevertheless, this group of plants is still widely unexplored in the scope of veterinary parasitology. In this context, the aims of this study were (1) to evaluate the *in vitro* anthelmintic properties of selected Mediterranean salt-tolerant plant species against L3 larvae exsheathment and egg hatching processes of *H. contortus* and *T. colubiformis;* (2) to explore the overall role of polyphenols in the anthelmintic activity, and (3) to compare the phytochemical composition determined by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n) of selected extracts, treated or not with poly-(poly)vinylpolirrilodine (PVPP), a polyphenol-binding agent.

3.2. MATERIAL AND METHODS

3.2.1. Plant collection and processing

Plant species were selected based on the ethnopharmacological uses, phenolic content reported in the literature, availability/accessibility of the biomass, and/or unreported anthel- mintic properties. Thus, aerial parts of *Pistacia lentiscus* L. (Anacardiaceae), *Cladium mariscus* (L.) Pohl (Cyper- aceae), *Inula crithmoides* L. (Asteraceae), *Helichrysum italicum* (Roth) G. Don subsp. *picardii* (Boiss. & Reut.) Franco (Asteraceae), *Calystegia soldanella* (L.) R. Br. (Convolvulaceae), *Medicago marina* L. (Fabaceae), *Plantago coronopus* L. (Plantaginaceae), *Limoniastrum monopetalum* (L.) Boiss. (Plumbaginaceae), and *Crucianella maritima* L. (Rubiaceae; Fig. 3.1) were collected in 4 districts of the Algarve coastal region (Southern Portugal), between 2017 and 2018 (Table 3.1). *Inula crithmoides*, *C. soldanella*, *M. marina*, *P. coronopus*, and *L. monopetalum* are halophyte plants included in the eHALOPH database while others such as *P. lentiscus*, *C. mariscus*, and *C. maritima* have recognized salt-tolerance despite not yet included in this database. After collection, samples were taken to the laboratory, washed, frozen at −20 °C, freeze-dried (Lyoalfa 15) for three days, and ground using a coffee and a ball miller (Retsch PM 100).

Mandatory licenses for the collection of all plant specimens from the wild in the Portuguese territory were obtained, and the collection protocol was performed according to the standard procedures recommended by "*Instituto da Conservação da Natureza e das Florestas* (ICNF)", the national regulatory body. The formal identification of the plant material was made by Dr. Luísa Custódio (CCMAR). Voucher specimens were kept in the XtremeBio group herbarium, at Centre of Marine Sciences (CCMAR), University of Algarve (UAlg), Faro, Portugal (Table 3.1).

3.2.2. Sample preparation

Dried ground samples were extracted with an 80% aqueous acetone solution (1:40, *w/v*), as previously used for the successful extraction of phenolic compounds and tannins from different salt-tolerant species (Jallali et al., 2014), at 20–25 °C, for 16 h, under stirring. Afterwards, the residue was filtered using a qualitative filter (Whatman no 4), and acetone was removed using a rotary evaporator under reduced pressure and

temperature (approximately 40 °C). The residue was later freeze-dried and recovered to be used in the *in vitro* anthelmintic assays.

Figure 3.1 Salt-tolerant species prospected from the Algarve region, Southern Portugal.

Table 3.1 Plant collection details, including collected organs, date, location and voucher number. Aerial organs: L, leaves; S, stems; FR, fruits; FL, flowers, I, inflorescences.

Species/family	Voucher No.	Aerial organs	Date	Location/coordinates
Helichrysum italicum subsp. *picardi* (Asteraceae)	XBH32	L/FL	Jul 2017	Tavira (37° 07′ 51.8″ N, 7° 36′ 37.6″ W)
Inula crithmoides (Asteraceae)	XBH04	L/S/FL	Oct 2017	Olhão (37° 01′ 11.7″ N, 7° 53′ 04.8″ W)
Pistacia lentiscus (Anacardiaceae)	XBH06	L/S/FR	Jan 2018	Portimão (37° 07′ 34.7″ N, 8° 36′ 02.3″ W)
Cladium mariscus (Cyperaceae)	XBH03	L/I	Jul 2017	Faro (37° 01′ 03.3″ N,7° 59′ 18.1″ W)
Calystegia soldanella (Convolvulaceae)	XBH07	L/S/FL	Apr 2018	Portimão (37° 07′ 23.1″ N, 8° 36′ 10.7″ W)
Medicago marina (Fabaceae)	XBH41	L/S/FL	Apr 2018	Portimão (37° 07′ 23.1″ N, 8° 36′ 10.7″ W)
Plantago coronopus (Plantaginaceae)	XBH02	L	Jan 2018	Olhão (37° 01′ 32.8″ N, 7° 53′ 04.4″ W)
Limoniastrum monopetalum (Plumbaginaceae)	XBH05	L/S	Jul 2017	Portimão (37° 07′ 34.7″ N, 8° 36′ 02.3″ W)
Crucianella maritima (Rubiaceae)	XBH40	L/S	Apr 2017	Portimão (37° 07′ 23.2″ N, 8° 36′ 12.3″ W)

3.2.3. Phenolic content of the extracts

Total phenolic content (TPC)

The TPC of the extracts was estimated using the Folin–Ciocalteau (F–C) reagent (Singleton et al., 1965). Briefly, 5 µL of the extracts (10 mg mL^{-1}) were added with 100 µL of the F–C reagent (1:10 in water, *v/v*) in 96-well plates, and left for 10 min at 20–25 °C, protected from light. After, it was added 100 µL of sodium carbonate (75 g L^{-1}, in water) and the plate incubated for 90 min in the dark. Absorbance was measured at 725 nm in a multiplate spectrophotometer reader (Biotek Synergy 4). A calibration curve was prepared using gallic acid as a standard. TPC was expressed as gallic acid equivalents (GAE; mg GAE g $extract^{-1}$, dry weight (DW)).

Total flavonoid content (TFC)

TFC was determined by the aluminum chloride ($AlCl_3$) method (Quettier-Deleu et al., 2000), by mixing 50 µL of the extracts at 10 mg mL with 50 µL of 2% $AlCl_3$ in methanol and left to incubate for 10 min at 20–25 °C. Absorbance was measured at 415 nm in a multiplate spectrophotometer reader. A calibration curve was prepared using quercetin as a standard. TFC was expressed as quercetin equivalents (QE; mg QE g $extract^{-1}$, DW).

Condensed tannins content (CTC)

CTC was evaluated by the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA–HCl) colorimetric method (Li et al., 1996) adapted to 96-well microplates (Rodrigues et al., 2015). Ten microliters of the extracts (10 mg mL^{-1}) were mixed with 200 µL of a methanol solution of DMACA (1%, *w/v*), and 100 µL of hydrochloric acid

(37%, *v/v*). After a 15 min incubation period, absorbance was measured at 640 nm in a multiplate spectrophotometer reader. A calibration curve was prepared using catechin as a standard. The concentration of CT was expressed as catechin equivalents (mg CE g extract^{-1}, DW).

Chemical profiling by High-performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric detection (HPLC-ESI-MS^n)

HPLC-ESI-MS^n analyses were performed with an Agilent Series 1100 HPLC system with a G1315B diode array detector (Agilent Technologies), and an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics) with an electrospray interface. Separation was performed in a Luna Omega Polar C_{18} analytical column (150×3.0 mm; 5 µm particle size) with a Polar C_{18} Security Guard cartridge (4×3.0 mm), both purchased from Phenomenex. Detailed chromatographic conditions are available in Supplementary Material files. Compounds' identification was performed by mass spectrometry data. Compounds' quantitation was carried out by UV using analytical standards of neochlorogenic acid (320 nm), chlorogenic acid (320 nm), protocatechuic acid (280 nm), catechin (280 nm), sinapic acid (320 nm), ferulic acid (320 nm), quercetin (350 nm), apigenin (350 nm), and kaempferol (350 nm). Detection limits (3σ criterion) ranged between 0.06 and 0.15 mg L^{-1}. Calibration graphs were constructed in the 0.5–100 mg L^{-1} range. Peak areas at the corresponding wavelengths were plotted versus analyte concentration. Each analytical standard was used to quantify the corresponding compounds or compounds of the same chemical family for which the exact analytical standards were not available. Repeatability (n=10) and intermediate precision (n=9, three consecutive days) were lower than 4 and 8%, respectively. The robustness of the chromatographic method was evaluated by recording analyte signals at ± 2 nm of the optimum wavelength and by slightly varying the percentage of the mobile phase (2% changes), observing variations lower than 5% for all the analytes concerning the optimum conditions.

3.2.4. *In vitro* anthelmintic assays

Haemonchus contortus *and* Trichostrongylus colubriformis *parasites*

Third-stage larvae (L3) and eggs were obtained from faeces of monospecifically infected caprine and ovine donors, with susceptible strains of *H. contortus* and *T. colubriformis*. L3 larvae had been maintained in culture flasks for 1 month, at 4 °C, before use in the

Larval Exsheathment Inhibition Assay (LEIA), while eggs were collected on the day of the Egg Hatching Inhibition Assay (EHIA) and used up to 2 h after collection (Jackson & Hoste, 2010).

Larval Exsheathment Inhibition Assay (LEIA)

LEIA was performed as previously described by Bahuaud and colleagues (2006). The extracts were diluted in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH 7.2), and incubated with L3 larvae (approx. 800 larvae per mL) at 23 °C for 3 h. Afterwards, larvae were washed and centrifuged with PBS 3 times, and the pellet resuspended in 200 µL of PBS. To initiate the LEIA, 40 µL of the test solution was used to count the proportion of ensheathed/exsheathed larvae at 0 min. The remaining larvae (160 µL) were then subjected to an artificially induced exsheathment by exposure to a solution of Milton (2% *w/v* sodium hypochlorite, 16.5% *w/v* sodium chloride) diluted in PBS. Milton optimal concentration was determined for each batch before use in order to ensure a gradual exhsheathment process, reaching 100% exsheathment in 60 min. After 20-, 40-, and 60-min exposure, the number of ensheathed and exsheathed larvae were counted under a microscope (400×). Four replicates were performed for each extract concentration, and the negative control, PBS, was run in parallel. Percentage of larvae exsheathment (LE) for each replicate was calculated according to the following formula: %LE = [(number of exsheathed larvae)/(number of exsheathed + ensheathed larvae) × 100].

Egg Hatching Inhibition Assay (EHIA)

Faeces material was filtrated using a gaze hydrophyle compress for 2 times, transferred to a 25 µm sieve, and washed with distilled water. The residue was centrifuged three times using a saline saturated solution (d = 1.2) to concentrate the eggs, and the pellets were recovered in PBS for use in the experiments. Afterwards, the eggs were quantified, plated in 48-well sterile plates (100 eggs per well), and exposed to the extracts at concentrations ranging from 5000 to 78 µg mL^{-1} in PBS. Plates were incubated at 27 °C for 48 h in the dark, and the number of larvae and eggs, in each well, was registered after microscopic counting. Six replicates were performed for each extract concentration, and the negative PBS control was run in parallel. The percentage of egg hatching (EH) for each well was

calculated according to the following formula: % EH = [(number of larvae)/(number of eggs + larvae) × 100].

3.2.5. Polyvinylpolypyrrolidone (PVPP) treatment

PVPP is a polyphenol inhibitor that binds to tannins and flavonoids, removing these metabolites from the solution (Doner et al., 1996). To ascertain the role of the polyphenols in the anthelmintic activity of the extracts, PVPP was added at a ratio of 50:1 to the active ones, respectively for eggs and larvae assays, in PBS, and incubated overnight at 4 °C. The maximum concentration tested for LEIA was 1200 µg mL^{-1}; for EHIA it was 2500 µg mL^{-1}. Thereafter, the samples were centrifuged for 10 min at 4500 rpm, and the supernatant depleted in polyphenols was tested in LEIA and EHIA assays. The extracts exposed or not to PVPP plus a negative control (PBS) were run in parallel.

3.2.6. Statistical analyses

At least four replicates per concentration were included in all experiments. The results on phenolic content are expressed as mean ± standard error of the mean (SEM). Anthelmintic data are expressed as the concentrations inhibiting 50% of larval exsheathment or egg hatching (IC_{50} values, µg mL^{-1}), and 95% confidence intervals (CI), obtained by Probit analysis. SPSS Statistics v. 26.0 software was used to assess significant differences among IC_{50} values, through relative median potency estimates, and among phenolic data, by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey HSD test. Spearman correlations were calculated between the total flavonoids, total phenols, and the IC_{50} values for LEIA on the 2 nematode species.

3.3. RESULTS AND DISCUSSION

3.3.1. Total Phenolics, Total Flavonoids and Condensed Tannins contents

The phenolic content of the extracts is presented in Table 3.2. The total phenolic content of all species ranged between 14.2 and 226.3 mg GAE eq. g^{-1} DW extract while the total flavonoid content ranged between 13.3 and 45.4 mg QE g^{-1} DW. Lopes and colleagues (2016) reported higher TPC values for *C. mariscus* (254 mg GAE g^{-1} DW), *C. soldanela* (144 mg GAE g^{-1} DW), *I. crithmoides* (141 mg GAE g^{-1} DW), *L. monopetalum* (248 mg GAE g^{-1} DW) 80% acetone water extracts, except for *P. lentiscus* (130 mg g^{-1} DW), but lower flavonoid contents in comparison to our work (1.26–13.8 mg rutin g^{-1} DW). In another work, *H. italicum picardii* infusions and decoctions of aerial organs have been previously described as rich sources of flavonoids (91.8–119 mg rutin 200 mL^{-1}; Pereira et al., 2017a). Moreover, a lower combined TPC value was detected in *P. coronopus* leaves and flowers extracts of different polarities (72.1 mg GAE g^{-1} DW) but increased TFC levels (282.8 mg rutin g^{-1} DW; Pereira et al., 2017b). In this study, total condensed tannins were detected only in three species, in the following concentration order: *P. lentiscus*>*L. monopetalum*>*C. mariscus*. In agreement, tannins were formerly detected in the same three formerly mentioned species, although at lower concentrations (6.63–38.7 mg CE g^{-1} DW, extract; Lopes et al., 2016). It is worth mentioning that dissimilarities between our results and those of other authors may be the reflection of different extraction methodologies and standards employed as well as environmental and plant-related factors.

Table 3.2 Phenolic content of acetone water extracts of selected plant species. *n.d.* not detected, *TPC* total phenolic content, expressed as mg gallic acid equivalents g^{-1} extract (mg GAE g^{-1}, DW), *TFC* total flavonoid content, expressed as mg quercetin equivalents g^{-1} extract (mg QE g^{-1}, DW), *CTC* condensed tannins content, expressed as mg catechin equivalents g^{-1} extract (mg CE g^{-1}, DW). Values are expressed as mean with standard deviation of the mean represented. *Data published in (Oliveira et al., 2021b). Different letters superscript represents significant differences among species, for each assay ($p < 0.05$; Tukey HSD).

Species	TPC	TFC	CTC
Helichrysum italicum subsp. *picardi*	83.7 ± 0.6^{e}	45.4 ± 1.3^{a}	n.d.
Inula crithmoides	27.2 ± 1.1^{g}	13.3 ± 0.1^{g}	n.d.
Pistacia lentiscus	226.3 ± 0.8^{a}	28.9 ± 0.4^{c}	607.3 ± 29.4^{a}
Calystegia soldanella	73.2 ± 0.8^{f}	42.0 ± 1.0^{b}	n.d.
Cladium mariscus	$112.3 \pm 2.1^{*c}$	$18.5 \pm 0.4^{*ef}$	$153.1 \pm 2.2^{*c}$
Medicago marina	14.2 ± 0.5^{h}	27.0 ± 0.8^{cd}	n.d.
Plantago coronopus	160.0 ± 3.0^{b}	25.2 ± 0.5^{d}	n.d.
Limoniastrum monopetalum	96.7 ± 2.9^{d}	16.0 ± 0.3^{fg}	281.4 ± 22.6^{b}
Crucianella maritima	25.5 ± 0.9^{g}	20.4 ± 0.2^{e}	n.d.

3.3.2. *In vitro* anthelmintic properties

Table 3.3 summarizes the results of the *in vitro* activity of salt-tolerant plant extracts against *H. contortus* L3 larvae and eggs and *T. colubriformis* L3 larvae and eggs obtained in LEIA and EHIA assays. Lentisk (*P. lentiscus*) exhibited the highest activity on LEIA (IC_{50} = 27.8–29.7 μg mL^{-1}) and egg hatching processes (IC_{50} = 197.7 and 223.9 μg mL^{-1}), without significant differences between GIN species. Lentisk is an evergreen shrub with high polyphenol content and previous results have shown both *in vitro* and *in vivo* anthelmintic properties (Manolaraki et al., 2010; Landau et al., 2010; Azaizeh et al., 2013; 2015). In previous studies, *P. lentiscus* extracts (acetone, ethanol and/or water) exhibited less than 20% larvae exsheathment and migration at 1200 μg mL^{-1} (Manolaraki et al., 2010; Landau et al., 2010; Azaizeh et al., 2013). Nevertheless, the results for the *in vitro* egg hatching assay are herein, to the best of our knowledge, described for the first time. Following *P. lentiscus*, *L. monopetalum, C. mariscus* and *H. italicum. picardii* extracts exhibited the most promising results towards both GIN species and life stages (Table 3.3). *Limoniastrum monopetalum* is a highly salt- tolerant shrub, widely distributed in the Mediterranean area, and was as effective as *P. lentiscus* in LEIA ($p < 0.05$), with IC_{50} values lower than 50 μg mL^{-1} (no significant difference between the two tested parasites; $p > 0.05$). In EHIA, *L. monopetalum* was also the most active species, besides *P. lentiscus*, with similar activity towards both parasites (IC_{50} = 1999.9 and 2102.5 μg mL^{-1}, respectively).

Table 3.3 *In vitro* anthelmintic activity of acetone extracts of selected plants on *H. contortus* and *T. colubriformis*, by L3 larvae exsheathment (LEIA) and egg hatching assays (EHIA). Results are expressed as IC_{50} values (μg mL^{-1}) and 95% confidence intervals in brackets. *n.d.* not determined since IC_{50} is higher than 5000 μg mL^{-1}. Capital and small letters represent significant statistical differences among botanical species (rows) and GIN species (columns) for each assay, respectively, based on Relative Median Potency Estimates.

	LEIA		EHIA	
Species	***H. contortus***	***T. colubriformis***	***H. contortus***	***T. colubriformis***
Helichrysum italicum subsp. *picardi*	92.8^{Ab} (78.9–107.4)	132.5^{Bcd} (112.0–157.1)	2947.7^{Ac} (2772.5–3136.1)	3707.5^{Bd} (3494.4–3941.5)
Inula crithmoides	300.8^{Ac} (231.5–391.2)	1030.8^{Be} (731.3–1563.0)	n.d.	n.d.
Pistacia lentiscus	27.8^{Aa} (21.3–36.8)	29.7^{Aa} (22.2–39.7)	197.7^{Aa} (158.3–243.8)	223.9^{Aa} (185.0–268.7)
Calystegia soldanella	270.6^{Ac} (204.9–368.2)	270.8^{Ad} (197.7–384.4)	n.d.	n.d.
Cladium mariscus	88.9^{Ab} (66.3–118.7)	77.8^{Abc} (60.6–100.0)	1496.6^{Ab} (1326.5–1698.9)	2575.5^{Bc} (2324.1–2881.8)
Medicago marina	222.6^{Ac} (179.8–278.6)	211.2^{Ad} (159.7–282.2)	n.d.	3860.5^{d} (3501.6–4343.8)
Plantago coronopus	94.0^{Ab} (71.6–121.2)	212.4^{Bd} (156.3–292.6)	n.d.	n.d.
Limoniastrum monopetalum	39.4^{Aa} (33.2–46.4)	47.9^{Aab} (37.1–60.4)	1999.9^{Ab} (1693.6–2408.2)	2102.5^{Ab} (1813.2–2477.8)
Crucianella maritima	447.2^{Ad} (302.5–707.7)	1024.5^{Be} (616.9–2153.1)	n.d.	n.d.

Cladium mariscus, or sawgrass, is an evergreen grass-like plant occurring in coastal saltmarshes in the Mediterranean region. *C. mariscus* extract inhibited L3 larvae exsheathment (IC_{50} = 77.8–88.9 μg mL^{-1}), without significant differences between both parasite species ($p > 0.05$). In contrast, in the EHIA, *C. mariscus* was more effective towards *H. contortus* (IC_{50} = 1496.6 μg mL^{-1}) than *T. colubriformis* (IC_{50} = 2575.5 μg mL^{-1}; $p < 0.05$). *Helichrysum italicum* subsp. *picardii* (everlasting) is an aromatic salt tolerant plant commonly found in sandy soils, such as sand dunes, along the Southern European coast. Everlasting extract exhibited IC_{50} values ranging between 92.8–132.5 μg mL^{-1} on LEIA, and 2947.7–3707.5 μg mL^{-1} on EHIA. Interestingly, *H. contortus* larvae and eggs were more susceptible to the *H. italicum picardii* extract than those of *T. colubriformis* ($p < 0.05$).

It is well recognized that the anthelmintic activity is affected by the class, structure and concentration of secondary metabolites (Hoste et al., 2015). Moreover, these metabolites have different effects, depending on the target parasite species and life development stages (Hoste et al., 2015). A higher susceptibility of *H. contortus* in comparison to *T. colubriformis*, as observed for *C. mariscus* and *H. italicum picardii* extracts, has been previously documented for other bioactive plants, such as sainfoin, and individual chemical structures, depending on the ratios of prodelphinidins/procyanidins (Paolini et al., 2004; Brunet et al., 2006; Quijada et al., 2015). The authors suggest that such

differences can reflect dissimilarities on the composition of specific parasite sheath proteins, that interact differently with the chemical groups (Brunet et al., 2006; Quijada et al., 2015). The same conclusion can be driven for differences among parasite stages, as the eggshell and larvae coat differ in their structural components, which has also been recorded with conventional anthelmintic drugs (Mansfield et al., 1992; Hoste et al., 2015). This may explain the results obtained for *P. coronopus,* which was more active against larvae exsheathment (IC_{50} = 94.0 and 212.4 μg mL^{-1}), and inactive towards eggs, of both parasite species, at the maximum concentration tested. Overall, IC_{50} results obtained in LEIA are frequently reported as lower than EHA, suggesting that infective L3 larvae are more susceptible than eggs (Oliveira et al., 2017; Zabré et al., 2017).

Calystegia soldanella, *C. maritima* and *M. marina* co-occur in sand dunes along the Algarve coastline, while *I. crithmoides* can be found in highly saline environments, such as salt marshes. These four species were mildly to poorly active on both assays (Table 3.3). Interestingly, while *I. crithmoides* was mostly ineffective in this study, its related species, *I. viscosa* 70% ethanolic extract exhibit anthelmintic properties against the larvae exsheathment of a mixture of *Teladorsagia circumcincta* and *T. colubriformis* parasites (Azaizeh et al., 2015), suggesting significant chemical diversity among the genus.

Overall, the nine plant extracts had comparable effects between the two GIN species (Spearmen correlation; $R^2 = 0.96$; $p < 0.01$). In addition, a negative correlation between the total phenolic content and the anthelmintic activity was noted, particularly with *H. contortus* parasites (Spearmen correlation; $R^2 = 0.783$; $p < 0.05$), suggesting that these metabolites may be involved in the antiparasitic nematode's effects.

3.3.3. Role of polyphenols in the anthelmintic activity: PVPP as a polyphenol binding agent

In order to ascertain the role of polyphenols in the anthelmintic properties, the four plant extracts presenting results for both LEIA and EHIA were selected for further studies using PVPP. PVPP is a polyphenol inhibitor, as it binds to tannins and flavonoids, removing these metabolites from the solution (Doner et al., 1993). Thus, if after PVPP exposure a loss of the anthelmintic activity is observed, it can be assumed that polyphenols are most probably responsible for the activity once they were formerly removed.

The effects of the addition of PVPP to extracts on EHIA and LEIA are illustrated in Figs. 3.2 and 3.3, respectively. The application of all the extracts with PVPP largely restored the egg hatching process (Fig. 3.2) to control values, suggesting that polyphenols are most probably involved in the inhibition of this life stage development. Vargas-Magaña and colleagues (2014), while exploring the role of polyphenols on the anthelmintic effects of several extracts of tannin-containing tropical plants on EHIA, concluded that the main mechanism of action was by impairing larvae eclosion from the eggs. Likewise, we noted a high number of larvae trapped inside the eggs after the application of these active extracts (data not shown).

In contrast to EHIA, results with PVPP varied on LEIA (Fig. 3.3): the application of the *L. monopetalum* extract, resulted in 60–70% of larvae exsheathment of both parasite species after PVPP addition for 60 min, in contrast to 0% in the non-treated sample; the extract from *H. italicum picardii* pre-incubated with PVPP remained mostly completely active. Subtle changes were observed for *C. mariscus* (approx. 20–40% of larvae exsheathment after 60 min of treatment) for both parasite species, while *P. lentiscus* had only around 20% of larvae exsheathment at 60 min, after PVPP treatment. These results suggest that other bioactive metabolites, alone or in synergy, can be present in all extracts tested, especially for *H. italicum picardii*, *P. lentiscus*, and *C. mariscus*. In agreement with our results, other authors already reported that *P. lentiscus* extracts remain active on GIN larvae migration after exposure to PVPP (Manolaraki et al., 2010).

The remaining activity on LEIA for the majority of the extracts tested should be carefully analyzed, and two scientific questions arise. First, was the ratio of PVPP used insufficient to cope with the high phenolic content of the extracts? Despite being commonly used, Manoloraki et al. (2010) questioned this hypothesis when testing *P.lentiscus* for larvae migration after PVPP addition, since this species has a high polyphenol content, comparable to our results. On the other hand, are other bioactive metabolites present in the extracts that are also effective in inhibiting larvae exsheathment? For instance, different authors suggest that terpenes may be responsible for the remaining *in vitro* and *in vivo* anthelmintic properties of *P. lentiscus* after the addition of PVPP or polyethylene glycol (PEG), a similar inhibitor of polyphenols (Manolaraki et al., 2010; Landau et al., 2010).

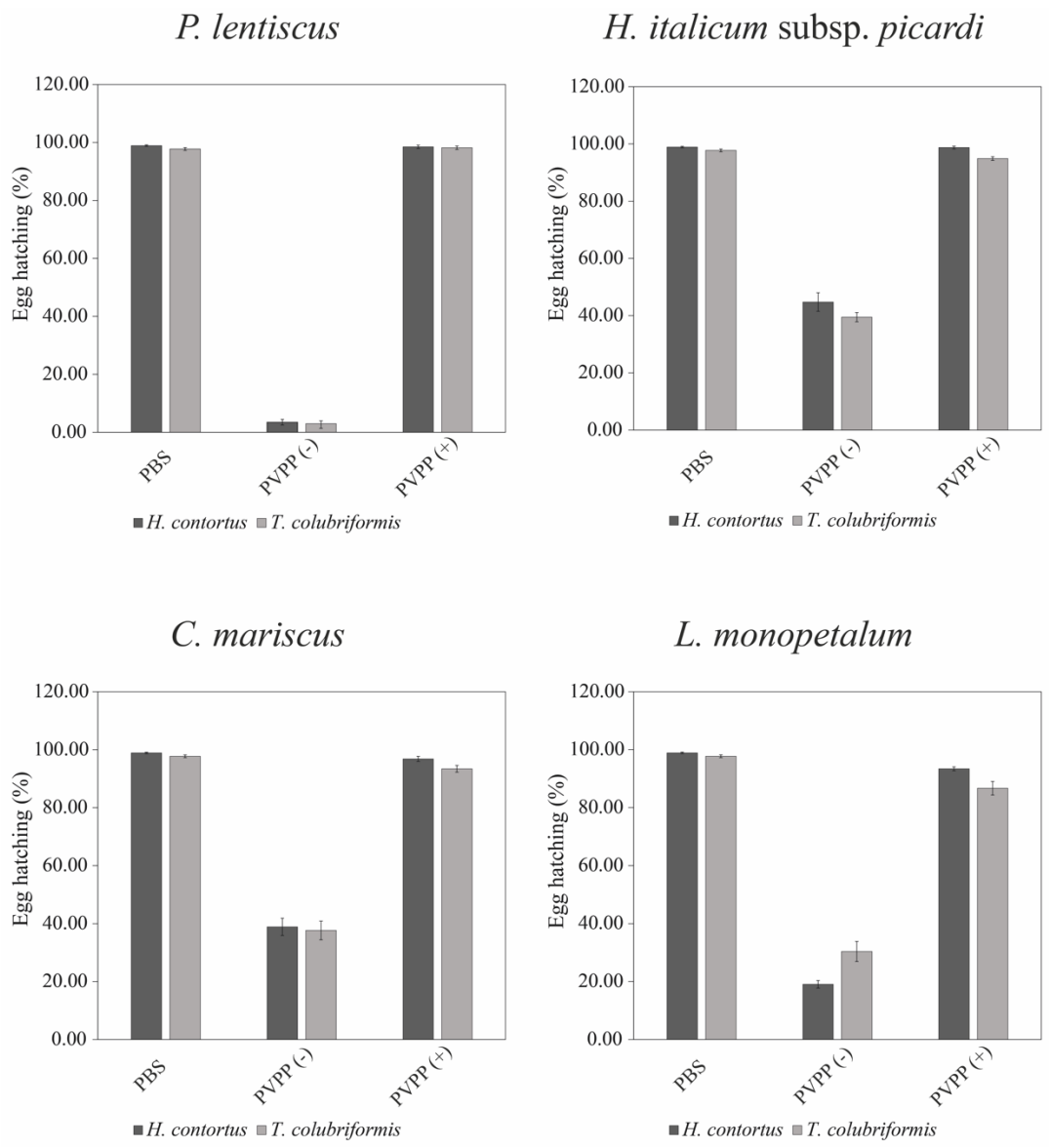

Figure 3.2 Effect of the application of PVPP on extracts of 4 selected plants, on the egg hatching inhibitory assay (EHIA) for *H. contortus* and *T. colubriformis* at concentration of 2500 µg mL^{-1}, either treated [PVPP(+)] or not [PVPP(−)] with PVPP.

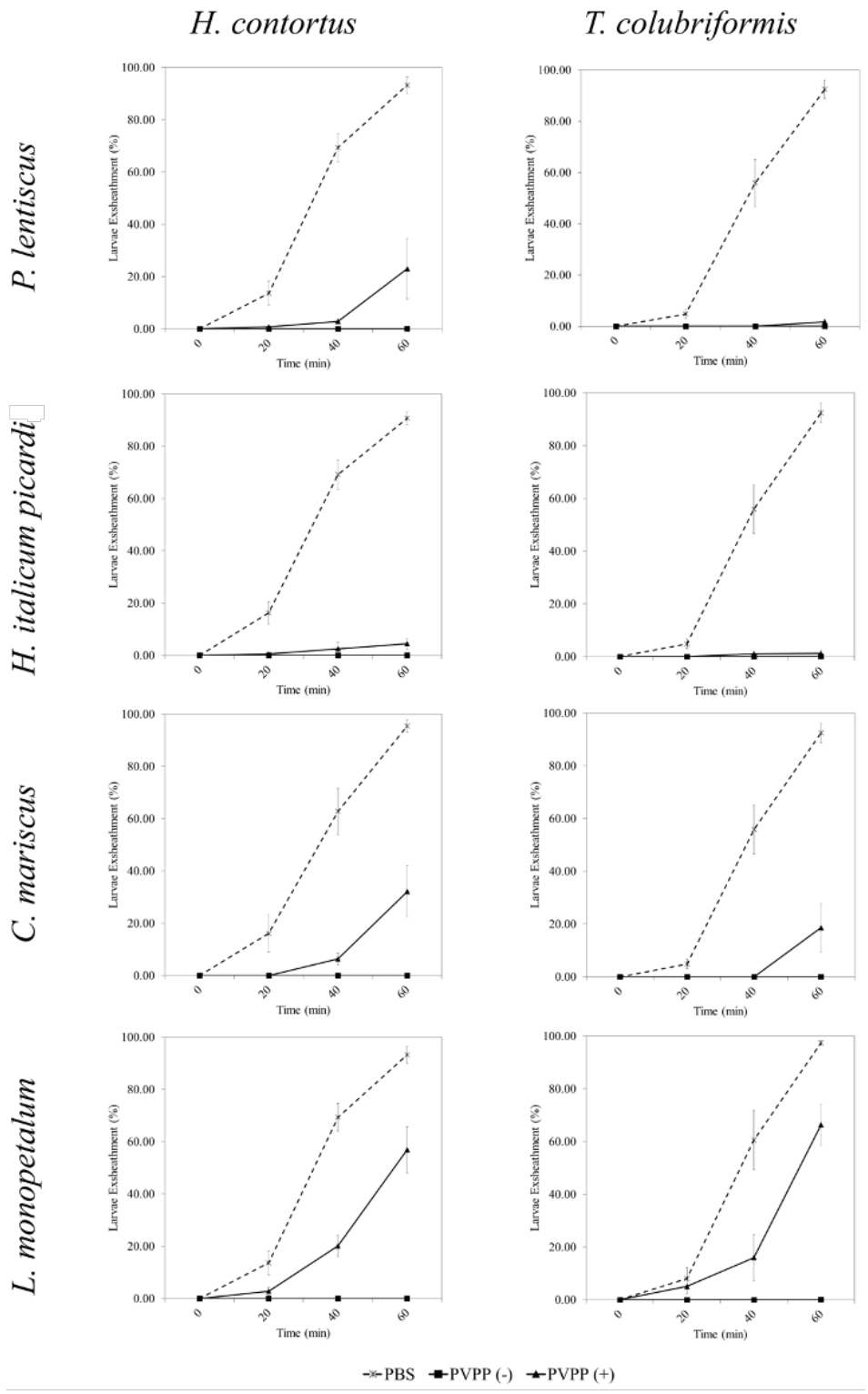

Figure 3.3 Effect of the application of PVPP on extracts of 4 selected plants, on L3 larvae exsheathment assay (LEIA) for *H. contortus* and *T. colubriformis* at concentration of 2500 µg mL^{-1}, either treated [PVPP(+)] or not [PVPP(−)] with PVPP.

Additionally, Botura and colleagues (2013) described that the flavonoid fraction of *Agave sisalana* Perrine (sisal) had higher activity on egg hatching, while the saponin fraction

had mostly larvicidal effects. In an attempt to address these scientific questions, and elucidate the possible metabolites involved, we have conducted an HPLC-ESI-MS^n comparative analysis on the active samples, before and after PVPP treatment.

3.3.4. HPLC-ESI-MS^n comparative analysis of the chemical profile of non-treated and treated-PVPP samples

The HPLC-ESI-MS^n analysis was performed in the most active extracts, with and without PVPP. Obtained chromatograms are represented in Fig. 3.4 while the chemical profile of each species is depicted in Tables 3.4, 3.5, 3.6 and 3.7. The characterization of the compounds is detailed in Supplementary Material files.

The main constituents of *P. lentiscus* extract were flavonoid glycosides (mainly from myricetin and quercetin; approx. 53 mg g^{-1} DW), and galloylquinic acid and di-*O*-galloylquinic acid isomers (60 mg g^{-1} DW; Table 3.4; Suppl. files, Table I). In agreement with our findings, Romani et al. (2002) detected a high concentration of galloyl derivatives (5.3% DW), and a substantial amount of myricetin and quercetin glycosides (1.5% DW), extracted from a 70% ethanol solution of leaves. Hydrolysable tannins are a group of gallic acid esters associated with polyols (*e.g.,* glucose, glucitol, quinic acid), and the etherification or oxidation of the galloyl groups leads to the formation of complex structures (gallotannins and ellagitannins; Castañeda et al., 2012). Plant extracts containing hydrolysable tannins with gallic acid units were more effective as anthelmintics than those containing condensed tannins (Katiki et al., 2013). Nevertheless, the oligomerization and molecular weight of tannins may affect the anthelmintic activity, as is the case, for example, of elagitannins and condensed tannins (Quijada et al., 2015; Karonen et al., 2020). Other metabolites present in lower concentrations in *P. lentiscus* extract with reported anthelmintic effects include flavan-3-ols and its galloyl derivatives, namely epigallocatechin (6.4 mg g^{-1} DW), gallocatechin gallate (6.8 mg g^{-1} DW) and catechin (5.0 mg g^{-1} DW). Molan et al. (2003) found that the presence of the galloyl group on flavan-3-ols was crucial for the activity on *T. colubrifomis* egg hatching (20% vs. 100% inhibition at 1 mg mL^{-1}), and also more effective on immobilizing infective larvae

(100% inhibition at 100–150 µg mL^{-1}; Molan et al., 2003).

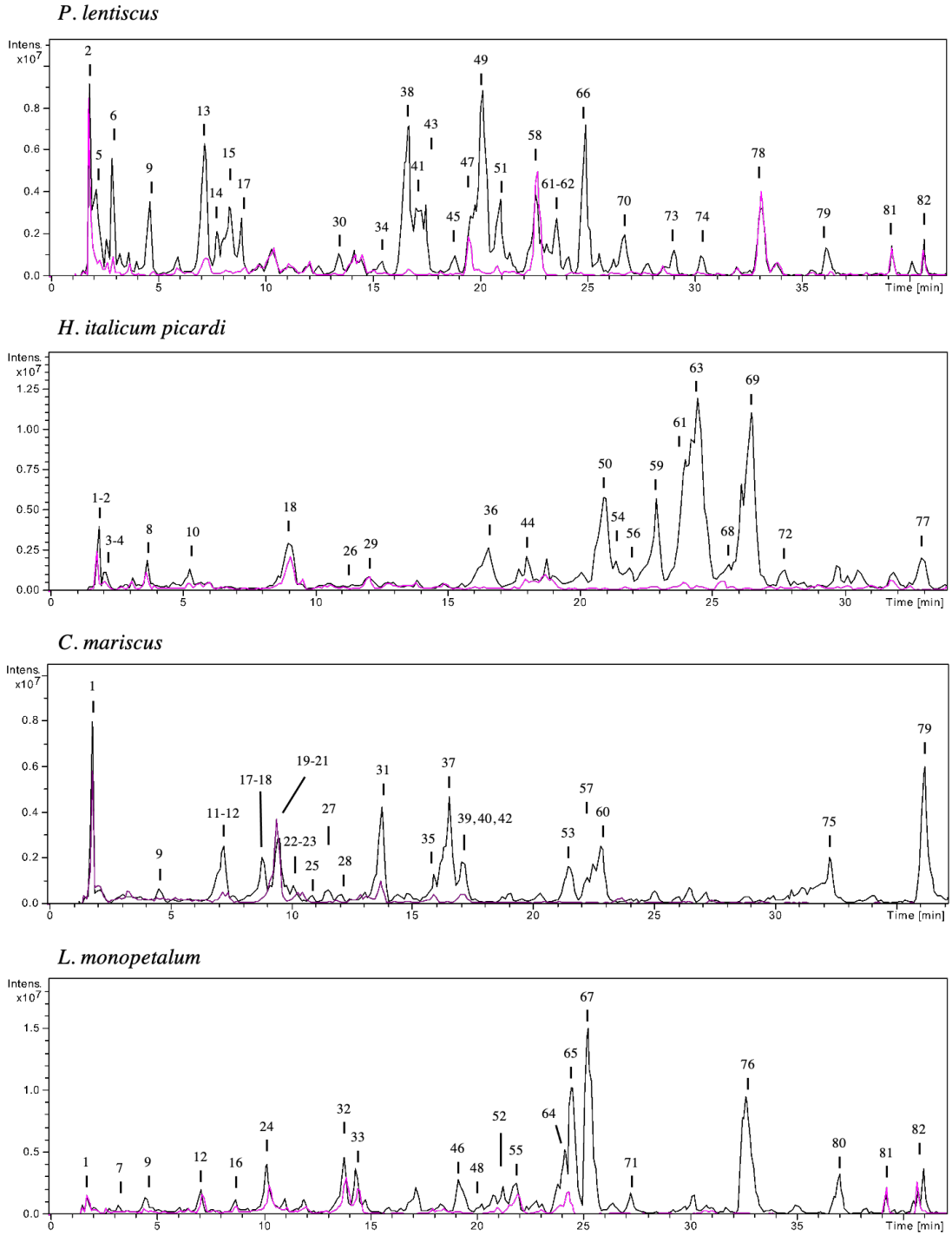

Figure 3.4 Base peak chromatogram of the extracts of 4 selected plants. The blackline represents the chromatogram of non-treated samples, while the pink line represents the chromatogram of PVPP-treated extracts, with numbers referring to the compounds described in Tables 3.4., 3.5., 3.6, and 3.7.

Table 3.4 Chemical profile of the extract of *Pistacia lentiscus* aerial organs. Column "PVPP" indicates if the compound was also present in the corresponding extract treated with PVPP.

No.	Rt (min)	$[M-H]^-$ m/z	m/z (% base peak)	Assigned identification	PVPP
2	1.9	191	MS2 [191]: 173 (100)	Quinic acid	+
5	2.2	495	MS2 [495]: 343 (100), 325 (14), 169 (16)	Di-*O*-Galloylquinic acid	
			MS3 [495 → 343]: 191 (99), 169 (100), 125 (20)		
			MS4 [495 → 343 → 169]: 125 (100)		
6	2.9	343	MS2 [343]: 191 (100), 169 (15), 125 (4)	Galloylquinic acid	+
9	4.6	305	MS2 [305]: 261 (31), 221 (35), 219 (71), 179 (100), 165 (38)	(Epi)gallocatechin	
13	7.2	495	MS2 [495]: 343 (100), 325 (7), 169 (13)	Di-*O*-Galloylquinic acid	
			MS3 [495 → 343]: 191 (100), 169 (77), 125 (10)		
14	7.8	495	MS2 [495]: 343 (100), 325 (36), 191 (12), 169 (15)	Di-*O*-Galloylquinic acid	
			MS3 [495 → 343]: 191 (40), 173 (9), 169 (100), 125 (10)		
15	8.4	183	MS2 [183]: 168 (100)	Methyl gallate	
			MS3 [183 → 168]: 124 (100)		
17	8.8	289	MS2 [289]: 245 (100), 205 (40), 203 (14), 179 (22), 151 (9)	Catechin	
30	13.4	457	MS2 [457]: 331 (22), 305 (21), 169 (100)	(Epi)gallocatechin gallate	
			MS3 [457 → 169]: 125 (100)		
34	15.5	631	MS2 [631]: 479 (100)	Myricetin-hexoside-gallate	
			MS3 [631 → 479]: 317 (100), 316 (93), 179 (10)		
			MS4 [631 → 479 → 317]: 271 (100), 179 (38)		
38	16.6	625	MS2 [625]: 317 (100), 316 (87)	Myricetin-*O*-rutinoside	+
			MS3 [625 → 317]: 271 (100), 179 (90), 151 (22)		
41	17.1	493	MS2 [493]: 317 (100)	Myricetin-*O*-glucuronide	
			MS3 [493 → 317]: 179 (100), 151 (29)		
43	17.5	479	MS2 [479]: 317 (100), 316 (97)	Myricetin-*O*-hexoside	
			MS3 [479 → 317]: 271 (100), 179 (66), 151 (12)		
45	18.8	615	MS2 [615]: 463 (100), 301 (42)	Quercetin-hexoside-gallate	
			MS3 [615 → 463]: 301 (100)		
			MS4 [615 → 463 → 301]: 179 (98), 151 (100)		
47	19.6	449	MS2 [449]: 317 (44), 316 (100)	Myricetin-*O*-pentoside	
			MS3 [449 → 316]: 271 (100), 179 (26)		
49	20.1	463	MS2 [463]: 317 (95), 316 (100)	Myricetin-*O*-deoxyhexoside	+
			MS3 [463 → 316]: 271 (100), 179 (80), 151 (20)		
51	20.9	463	MS2 [463]: 301 (100)	Quercetin-*O*-hexoside	
			MS3 [463 → 301]: 179 (100), 151 (50)		
58	22.6	373 (+)	MS2 [373]: 211 (100), 193 (34), 175 (16), 135 (22), 119 (14)	Hydroferuloylglucose	+
61	23.4	433	MS2 [433]: 301 (100)	Quercetin-*O*-pentoside	
			MS3 [433 → 301]: 271 (100), 179 (87), 151 (68)		
62	23.5	447	MS2 [447]: 285 (100)	Kaempferol-*O*-hexoside	
			MS3 [447 → 285]: 255 (100), 229 (37), 227 (33)		
66	24.8	447	MS2 [447]: 301 (100)	Quercetin-*O*-deoxyhexoside	+
			MS3 [447 → 301]: 179 (48), 151 (100)		
70	26.7	585	MS2 [585]: 301 (100)	Quercetin-pentoside-gallate	
			MS3 [585 → 301]: 179 (100), 151 (98)		
73	29.0	431	MS2 [431]: 285 (100)	Kaempferol-*O*-deoxyhexoside	+
			MS3 [431 → 285]: 257 (93), 255 (100), 241 (55), 229 (36)		
74	30.2	569	MS2 [569]: 285 (100)	Kaempferol-pentoside-gallate	
			MS3 [569 → 285]: 285 (100), 257 (37), 151 (86)		
78	33.0	507	MS2 [507]: 461 (100), 293 (36)	Unknown	+
79	36.0	285	MS2 [285]: 285 (100), 241 (23)	Luteolin	
81	39.1	327	MS2 [327]: 291 (24), 229 (100), 211 (25), 171 (89)	Oxo-dihydroxy-octadecenoic acid	+
82	40.6	329	MS2 [329]: 311 (31), 229 (96), 211 (100), 171 (60)	Trihydroxy-octadecenoic acid	+

Table 3.5 Characterization of the compounds present in the extract of *Helichrysum italicum picardii* aerial organs. Column "PVPP" indicates if the compound was also present in the corresponding *H. italicum picardii* treated PVPP sample.

No.	Rt (min)	$[M-H]^-$ *m/z*	m/z (% base peak)	Assigned identification	PVPP
1	1.8	377	MS^2 [377]: 341 (100) MS^3 [377 → 341]: 179 (100), 161 (95), 143 (34) MS^4 [377 → 341 → 179]: 143 (94), 119 (100)	Disaccharide (HCl adduct)	+
2	1.9	191	MS^2 [191]: 173 (48), 111 (100)	Quinic acid	+
3	2.1	315	MS^2 [315]: 153 (100) MS^3 [315 → 153]: 123 (100), 108 (49)	Dihydroxybenzoic acid-*O*-hexoside	+
4	2.1	353	MS^2 [353]: 191 (100), 179 (26), 135 (7)	Caffeoylquinic acid	+
8	3.7	315	MS^2 [315]: 153 (100) MS^3 [315 → 153]: 109 (100)	Dihydroxybenzoic acid-*O*-hexoside	+
10	5.3	353	MS^2 [353]: 191 (100), 179 (37), 135 (9)	Neochlorogenic acid	+
18	9.0	353	MS^2 [353]: 191 (100), 179 (4), 173 (5), 135 (3)	Chlorogenic acid	+
26	11.2	179	MS^2 [179]: 135 (100)	Caffeic acid	
29	12.2	609	MS^2 [609]: 447 (100), 285 (37) MS^3 [609 → 447]: 285 (46), 284 (100), 255 (50), 151 (20) MS^4 [609 → 447 → 285]: 255 (100), 243 (15), 227 (17)	Kaempferol-dihexoside	+
36	16.4	479	MS^2 [479]: 317 (100) MS^3 [479 → 317]: 317 (100), 203 (10), 195 (16), 165 (21)	Unidentified-*O*-hexoside	
44	18.0	515	MS^2 [515]: 353 (100), 191 (12) MS^3 [515 → 353]: 191 (100), 179 (44), 173 (13), 135 (13)	Dicaffeoylquinic acid	
50	20.8	463	MS^2 [463]: 301 (100) MS^3 [463 → 301]: 179 (24), 151 (100)	Quercetin-*O*-hexoside	
54	21.6	493	MS^2 [493]: 331 (100) MS^3 [493 → 331]: 316 (100)	Mearnsetin-*O*-hexoside	
56	22.2	477	MS^2 [477]: 315 (100), 314 (16) MS^3 [477 → 315]: 300 (100)	Isorhamnetin-*O*-hexoside	
59	22.7	515	MS^2 [515]: 353 (100), 179 (18), 173 (21) MS^3 [515 → 353]: 191 (48), 179 (62), 173 (100), 135 (10)	Dicaffeoylquinic acid	
61	23.4	433	MS^2 [433]: 301 (100), 271 (12) MS^3 [433 → 301]: 271 (68), 255 (100), 179 (18), 151 (55)	Quercetin-*O*-pentoside	
63	24.1	515	MS^2 [515]: 353 (100), 191 (7), 179 (3) MS^3 [515 → 353]: 191 (100), 179 (58), 135 (21)	Dicaffeoylquinic acid	+
68	25.4	431	MS^2 [431]: 269 (100) MS^3 [431 → 269]: 225 (100)	Apigenin-*O*-hexoside	+
69	26.5	515	MS^2 [515]: 353 (100), 179 (12), 173 (18) MS^3 [515 → 353]: 191 (13), 179 (68), 173 (100), 135 (15)	Dicaffeoylquinic acid	+
72	27.4	463	MS^2 [463]: 301 (100) MS^3 [463 → 301]: 179 (100), 151 (76)	Quercetin-*O*-hexoside	
77	32.7	609	MS^2 [609]: 463 (100), 301 (47) MS^3 [609 → 463]: 301 (100), 271 (4) MS^4 [609 → 463 → 301]: 179 (62), 151 (100)	Quercetin-*O*-deoxyhexoside-*O*-hexoside	

Table 3.6 Characterization of the compounds present in the extract of *Cladium mariscus* aerial organs. Column "PVPP" indicates if the compound was also present in the corresponding *C. mariscus* treated PVPP sample.

No.	Rt (min)	$[M-H]^-$ *m/z*	m/z (% base peak)	Assigned identification	PVPP
1	1.8	377	MS^2 [377]: 341 (100) MS^3 [377 → 341]: 179 (100), 161 (24), 143 (13), 119 (25), 113 (20)	Disaccharide (HCl adduct)	+
9	4.6	305	MS^2 [305]: 261 (7), 221 (43), 219 (72), 179 (100), 165 (35)	(Epi)gallocatechin	
11	7.0	577	MS^2 [577]: 451 (38), 425 (100), 407 (96), 305 (21), 289 (45), 287 (17)	Procyanidin dimer	
12	7.2	305	MS^2 [305]: 261 (12), 221 (55), 219 (77), 179 (100), 165 (26)	(Epi)gallocatechin	
17	8.8	289	MS^2 [289]: 245 (100), 205 (43), 203 (28), 179 (24)	Catechin	
18	9.0	353	MS^2 [353]: 191 (100), 179 (3), 173 (4), 135 (1)	Chlorogenic acid*	+
19	9.3	865	MS^2 [865]: 739 (54), 713 (41), 695 (100), 577 (52), 451 (29), 407 (54), 405 (23), 289(19), 287 (41)	Proanthocyanidin trimer	
20	9.5	429	MS^2 [429]: 267 (100) MS^3 [429 → 267]: 205 (100), 113 (82)	Unknown	+
21	9.9	577	MS^2 [577]: 451 (69), 441 (17), 425 (30), 305 (100), 289 (10), 287 (8)	Proanthocyanidin dimer	
22	10.1	865	MS^2 [865]: 739 (76), 695 (100), 577 (83), 451 (18), 407 (97), 287 (58)	Proanthocyanidin trimer	
23	10.1	561	MS^2 [561]: 543(18), 435 (58), 409 (73), 425 (46), 289 (100), 271 (41) MS^3 [561 → 289]: 245 (100), 205 (57), 203 (30)	Proanthocyanidin dimer	
25	10.9	577	MS^2 [577]: 451 (25), 441 (9), 425 (100), 407 (61), 305 (43), 289 (33), 287 (10)	Proanthocyanidin dimer	
27	11.5	577	MS^2 [577]: 451 (28), 425 (10), 305 (100), 289 (4), 287 (6)	Proanthocyanidin dimer	
28	12.1	289	MS^2 [289]: 245 (100), 205 (48), 203 (19), 179 (25), 161 (10)	Epicatechin	
31	13.7	579	MS^2 [579]: 561 (16), 519 (16), 489 (100), 459 (99), 429 (18), 399 (50), 369 (14)	Luteolin-*C*-hexoside-*C*-pentoside	+
35	15.9	563	MS^2 [563]: 545 (14), 503 (15), 473 (48), 443 (100), 383 (37), 353 (43)	Apigenin-*C*-hexoside-*C*-pentoside	+
37	16.5	447	MS^2 [447]: 429 (14), 357 (70), 327 (100), 285 (3)	Luteolin-6-*C*-glucoside (isoorientin)	+
39	17.0	461	MS^2 [461]: 341 (100), 313 (66), 298 (37)	Unknown	+
40	17.0	549	MS^2 [549]: 531 (12), 489 (26), 459 (100), 441 (13), 429 (10), 399 (64), 369 (25)	Luteolin 6-*C*-pentosyl-8-*C*-pentoside	+
42	17.3	563	MS^2 [563]: 503 (22), 473 (100), 443 (69), 383 (61), 353 (97)	Apigenin-*C*-hexoside-*C*-pentoside	+
53	21.4	447	MS^2 [447]: 285 (100) MS^3 [447 → 285]: 285 (100), 241 (47), 151 (10)	Kaempferol-*O*-hexoside	
57	22.2	417	MS^2 [417]: 399 (22), 357 (100), 327 (49) MS^3 [417 → 357]: 339 (100), 311 (24), 297 (82), 285 (93)	Luteolin-*C*-pentoside	
60	22.8	243	MS^2 [243]: 225 (100), 201 (50), 199 (23), 157 (20)	Unknown	
75	32.1	485	MS^2 [485]: 375 (100), 357 (13) MS^3 [485 → 375]: 357 (100), 333 (22), 265 (39)	Unknown	
79	36.0	285	MS^2 [285]: 285 (100), 267 (5), 243 (2), 241 (3)	Luteolin	

Table 3.7 Characterization of the compounds present in the extract of *Limoniastrum monopetalum* aerial organs. Column "PVPP" indicates if the compound was also present in the corresponding *L. monopetalum* treated PVPP sample.

No.	Rt (min)	$[M-H]^-$ *m/z*	m/z (% base peak)	Assigned identification	PVPP
1	1.8	377	MS^2 [377]: 341 (100)	Disaccharide (HCl adduct)	+
			MS^3 [377 → 341]: 179 (100), 161 (3), 143 (14), 119 (24), 113 (6)		
7	3.2	169	MS^2 [169]: 125 (100)	Gallic acid	
9	4.6	305	MS^2 [305]: 261 (21), 221 (53), 219 (57), 179 (100)	(Epi)gallocatechin	
12	7.2	305	MS^2 [305]: 261 (17), 221 (32), 219 (49), 179 (100), 165 (25)	(Epi)gallocatechin	
16	8.6	303	MS^2 [303]: 223 (100)	Sinapic acid sulfate	+
			MS^3 [303 → 223]: 208 (100), 179 (37), 164 (35), 149 (5)		
24	10.2	273	MS^2 [273]: 193 (100), 178 (17), 149 (38), 134 (7)	Ferulic acid sulfate	+
32	13.8	457	MS^2 [457]: 329 (100), 169 (31)	Gallic acid derivative	+
			MS^3 [457 → 169]: 125 (100)		
33	14.4	457	MS^2 [457]: 329 (100), 245 (26), 203 (23), 165 (24)	Unknown	+
			MS^3 [457 → 329]: 314 (100)		
46	19.1	252	MS^2 [252]: 212 (100), 204 (4)	Unknown	
48	19.8	609	MS^2 [609]: 301 (100)	Rutin	
			MS^3 [609 → 301]: 179 (100), 151 (78)		
52	21.2	477	MS^2 [477]: 301 (100)	Quercetin-*O*-glucuronide	
			MS^3 [477 → 301]: 179 (90), 151 (100)		
55	21.7	567	MS^2 [567]: 331 (100)	Unknown	
			MS^3 [567 → 331]: 316 (100), 179 (67), 151 (33)		
64	24.1	437	MS^2 [437]: 357 (100), 151 (52)	Pinoresinol	+
			MS^3 [437 → 357]: 342(5), 311 (6), 151 (100), 136 (24)		
65	24.4	395	MS^2 [395]: 315 (100)	Isorhamnetin sulfate	
			MS^3 [395 → 315]: 300 (100), 271 (8), 255 (13)		
67	25.2	425	MS^2 [425]: 345 (100)	Methylated flavonoid sulfate	
			MS^3 [425 → 345]: 330 (100), 315 (34)		
			MS^4 [425 → 345 → 330]: 315 (100), 285 (74)		
71	27.2	425	MS^2 [425]: 345 (100), 330 (15)	Methylated flavonoid sulfate	
			MS^3 [425 → 345]: 330 (100)		
			MS^4 [425 → 345 → 330]: 315 (100), 271 (10)		
76	32.5	439	MS^2 [439]: 359 (100)	Methylated flavonoid sulfate	+
			MS^3 [439 → 359]: 344 (100)		
			MS^4 [439 → 359 → 344]: 329 (100)		
80	36.9	439	MS^2 [439]: 359 (100)	Methylated flavonoid sulfate	
			MS^3 [439 → 359]: 344 (100), 329 (18)		
81	39.1	327	MS^2 [327]: 291 (27), 229 (100), 211 (70), 209 (44), 171 (77)	Oxo-dihydroxy-octadecenoic acid	+
82	40.6	329	MS^2 [329]: 311 (14), 229 (100), 211 (44), 171 (18)	Trihydroxy-octadecenoic acid	+

In *P. lentiscus* PVPP-treated samples, the concentration of flavonoid glycosides (0.17 mg g^{-1} DW) and galloylquinic acid (2.2 mg g^{-1} DW) drastically dropped (Suppl. files, Table SI), which may justify the restoration of the egg hatching. On the other hand, the presence of these compounds in lower concentrations may explain the remaining activity on larvae. Nevertheless, compounds **2**, **58,** and **78** remained in this sample, and may also account for the activity.

Caffeoylquinic and dicaffeoylquinic acids were the most abundant compounds in *H. italicum picardii* extract (150 mg g^{-1} DW), followed by quercetin-*O*-glucosides (approx. 31 mg g^{-1} DW; Table 3.5; Suppl. files, Table SII). These findings were expected, since previous works identified high contents of these metabolites in aerial organs of the same species (Gonçalves et al., 2015; Pereira et al., 2017). Borges and colleagues (2019) found a significant correlation between the phenylpropanoid content (particularly chlorogenic acid, 1,3-dicaffeoylquinic, and 3,5-dicaffeoylquinic acids), and the ovicidal activity of 17 plant extracts from Pantanal wetlands against *Haemonchus placei* (Borges et al., 2019). Additionally, chlorogenic acid exhibited an IC_{50} value of 92.4 μg mL^{-1} against L3 larvae exsheathment of *H. contortus* and was also effective on preventing larvae hatching from eggs (IC_{50} = 520.8 μg mL^{-1}; Mancilla-Montelongo et al., 2019). These results point out the potential of caffeoylquinic and dicaffeoylquinic acids to be the active metabolites of *H. italicum picardii* extracts. However, some *O*-glycosides are also present that may contribute to the detected activity. For example, Barrau and colleagues (2005) tested the activity of 3 flavonol glycosides (quercetin-3-*O*-rutinoside or rutin, kaempferol- 3-rutinoside or nicotiflorin, and isorhamnetin-3-rutinoside or narcissin), and all reduced the migration of *H. contortus* L3 larvae in 25–35% when applied at 1200 μg mL^{-1}.

In *H. italicum picardii* PVPP-treated sample, although in lower concentrations, caffeoylquinic and dicaffeoylquinic acids remained in solution (8.3 mg g^{-1} DW), from which chlorogenic acid was the main compound (6.3 mg g^{-1} DW; Suppl. files, Table II). The high activity observed for the extract from *H. italicum picardii* treated with PVPP on larvae exsheathment is most likely due to the high content of chlorogenic acid remaining in the sample (Mancilla-Montelongo et al., 2019). Still, other caffeoylquinic and dicaffeoylquinic acids are present (2 mg g^{-1} DW) that might also add to its effects. On the other hand, in EHIA the lower amount of these compounds in the PVPP-treated sample may have not be sufficient to inhibit egg hatching, since this process was completely

restored. In fact, Borges and colleagues (2019) suggest that the concentration of monomeric and dimeric chlorogenic acid derivatives that enter in contact with eggs seems to be determinant for the activity, as observed for *Melanthera latifolia* ethanolic extract that had low concentrations of these compounds and was considered inactive (up to 80% egg hatching at 50 mg mL^{-1}).

Cladium mariscus acetone water extracts were previously reported as a rich source of polyphenols, particularly tannins by spectrophotometric methods, and chlorogenic, ferulic, and syringic acids were detected in higher amounts, through HPLC–DAD analysis (Lopes et al., 2016; Oliveira et al., 2021). In agreement, in this study, *C. mariscus* extract was mainly composed of flavan-3-ols (epigallocatechin, catechin), proanthocyanidins (5.1 mg g^{-1} DW), luteolin, C-glycosyl luteolin, a kaempferol glucoside, and an apigenin flavone (9.5 mg g^{-1} DW; Table 3.6; Suppl. files, Table III). Flavan-3-ols and proanthocyanidins have well recognized anthelmintic effects (Molan et al., 2003; 2004), and therefore, they are most likely involved in the activity of *C. mariscus* extract. Also, the activity of the flavonoid luteolin on *H. contortus* larvae exsheathment has been previously established (IC_{50} = 17.1 and < 71.5 μM; Klongsiriwet et al., 2015). Interestingly, Klongsiriwet and colleagues (2015) found that luteolin, even at low concentrations (30 μM), displays synergistic effects with procyanidins, leading to a fivefold lower IC_{50} of the mixture in comparison to the procyanidin fraction alone (75.9 vs. 356 μg mL^{-1}). Having this in mind, the combination of proanthocyanidins and luteolin in *C. mariscus* extract could act synergistically in the inhibition of the egg hatching. Nevertheless, the activity on LEIA was only partially restored after PVPP addition (approx. 20–40% larvae exsheathment), *i.e.,* the remaining metabolites are still exhibiting anthelmintic properties. In PVPP-treated samples, mainly *C*-glycosyl flavones (1.07 mg g^{-1} DW) and, to a less extent chlorogenic acid, remained in solution while the catechin derivatives and luteolin were removed (Table 3.6; Suppl. files, Table III). As previously addressed, chlorogenic acid exhibits significant anthelmintic activity *in vitro* against *H. contortus* larvae exsheathment and egg hatching (Mancilla-Montelongo et al., 2019). Despite the activity described for luteolin, the investigation of the anthelmintic properties of its glycosides is lacking. In general, *C*-glycosyl flavones exhibit antioxidant and anti-inflammatory properties (Zeng et al., 2013), and two flavone-*C*-glycosides namely isoschaftoside and schaftoside shown strong toxicity (LC_{50} = 114.66 μg mL^{-1} and 323.09

μg mL^{-1}) against the plant-parasitic nematode *Meloidogyne* incognita (Du et al., 2011). Moreover, it is worth noticing that compounds **20** and **39** are still unidentified, although present in PVPP-treated samples.

Previous works identified several phenolic compounds in *L. monopetalum* extracts including gallic, vanillic, ferulic, syringic, p-hydroxybenzoic, protocatechuic, chlorogenic, and trans-cinnamic acids, and also quercetin, apigenin, amentoflavone, flavones, methyl gallate, and myricetin (Trabelsi et al., 2010; Bouzidi et al., 2016). In the current work, the main metabolites identified in *L. monopetalum* extract were epigallocatechin, phenolic acids and derivatives, isorhamnetin sulfate, pinoresinol, methylated flavonoids sulfate and two oxylipins (Table 3.7). However, some of the major compounds, namely the methylated flavonoids sulfate **67**, **71**, **76**, and **80** were not identified, as well as the minor metabolites **33**, **46**, and **55**. The production of sulfated metabolites by plants is pointed out as an evolutionary trait to thrive in aquatic saline habitats, and part of the plant heavy metal detoxification mechanism (Aquino et al., 2011; Cambrollé et al., 2013). Indeed, *L. monopetalum* is a halophytic and metal accumulator shrub that thrives in saltmarshes under harsh biotic and abiotic stresses (*e.g.,* tidal fluctuations, salinity, heavy metal soils, sunlight exposure, UV radiation). Sulfated phenolics were previously identified in other halophyte species, such as *Limonium caspium* (Willd.) Gams (Gadetskaya et al., 2015) and *Halimione portucaloides* (L.) Aellen (Vilela et al., 2014). The pharmacological interest in sulphated flavonoids increased in the last decades, mainly driven by its hydrophobic nature, and many reported biological activities, like anti-coagulant, anti-viral, antioxidant, anti-inflammatory, antimicrobial (Correia-da-Silva et al., 2014).

Besides epigallocatechin (9.46 mg g^{-1} DW), the concentration of isorhamnetin sulfate (**65**) was high in *L. monopetalum* (6.4 mg g^{-1} DW) as well as phenolic acids and its derivatives (10.3 mg mL^{-1} DW; **7**, **16**, **24**, **32**). Delgado-Nuñez and colleagues (2020) attributed the main anthelmintic effects of *Prosopis laevigata* Willd. M. Johnston to isorhamnetin, which caused 100% mortality on *H. contortus* eggs at the lowest concentration tested (700 μg mL^{-1}), being also effective towards larvae (IC_{50} = 2.07 mg mL^{-1}). The glycoside isorhamnetin-3-rutinoside decreased *H. contortus* L3 migration by 35% at 120 μg mL^{-1} (Barrau et al., 2005). However, the activity of its sulfate structure is not reported. Among different classes of phenolic compounds, phenolic acids (*i.e.,* caffeic

acid, ferulic acid, and gallic acid) were the most potent anthelmintic metabolites against both *H. contortus* egg hatching (IC_{50} values = 0.56–4.93 μg mL^{-1}) and larval development (IC_{50} = 22–33 μg mL^{-1}; Sprengel-Lima et al., 2021). Nevertheless, one should keep in mind that structural modifications, such as glycosylation, methylation, and sulfation, may affect the bioactivity observed. For example, the substitution by a sugar unit in the quercetin structure showed a twofold increase in the larvicidal activity of rutin (Sprengel-Lima et al., 2021). Still, studies concerning the anthelmintic effects of sulphated phenolics are missing. Since these metabolites are the main suspects as bioactive components of *L. monopetalum* extract, it would be interesting for further works to be conducted, not only confirming the anthelmintic effects of isolated compounds but also clarifying the role of sulfate in structure–activity relationship studies.

After PVPP treatment, the activity of *L. monopetalum* extract on larvae exsheathment was restored by approximately 60–70% to the control values. Although the remaining compounds may have contributed to the overall activity, the major anthelmintic effects were annulated. As some main metabolites of *L. monopetalum* (**67**, **71**, **76**, **80**) remain to be identified and quantified, further studies on this species are required to completely understand its bioactive compound(s) and related anthelmintic properties.

3.4. CONCLUDING REMARKS

Due to the constant diffusion of resistance to synthetic anthelmintics in worm populations, the search for plants with antiparasitic activities and their bioactive metabolites that can be used for integrated control approaches of GIN, has expanded over the last 20 years (French et al., 2018). Extremophile plants, in particular salt-tolerant species, may represent an untapped reservoir of anthelmintic compounds for such purpose. To the best of our knowledge, this study explores for the first time the *in vitro* anthelmintic properties of eight salt-tolerant species, namely *H. italicum* subsp. *picardii*, *I. crithmoides*, *C. soldanela*, *C. mariscus*, *M. marina*, *P. coronopus*, *L. monopetalum*, and *C. maritima*, against two GIN species and life stages. *Pistacia lentiscus*, *L. monopetalum*, *C. mariscus*, and *H. italicum* subps. *picardii* were the most active against both parasite species and life stages (eggs and L3) targeted. The comparative HPLC-ESI-MS^n analysis coupled with the use of PVPP unraveled that different bioactive metabolites may be involved in the anthelmintic properties: flavonoid glycosides and galloylquinic acid

isomers in *P. lentiscus*; caffeoylquinic and dicaffeoylquinic acids and quercetin glycosides in *H. italicum picardii*; proanthocyanins, phenolic acids, and luteolin in *C. mariscus*; and sulphated and/or methylated flavonoids in *L. monopetalum*. Further work should be pursued to complete the identification of the main metabolites of *L. monopetalum*, since this species exhibited the most promising results after *P. lentiscus*. As recently comprehensively reviewed by Spiegler et al. (2017) and Liu and colleagues (2020), polyphenols have been the most extensively studied compounds regarding their anthelmintic effects, but the number of other individual phenolic compounds and their structural diversity investigated is still limited, particularly towards these two GIN species. Therefore, future work should focus on fully elucidating the activity of the main potential bioactive metabolites identified in this work, either alone and/or in synergy, and provide information on structure–activity effects. Still, the results obtained in this study for *L. monopetalum*, *C. mariscus*, and *H. italicum* subsp. *picardii* warrant further investigations on the potential use of these species either as nutraceutical and/or phytotherapeutic options and/or as sources of anthelmintic compounds against GIN in ruminants.

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SUPPLEMENTARY FILES

Characterization of compounds by HPLC-ESI-MS^n

The characterization of the phytochemicals was carried out by HPLC-ESI-MS^n using the negative ion mode, except for compound **58** (positive ion mode). Identification was performed using analytical standards – apigenin, caffeic acid, catechin, chlorogenic acid, ferulic acid, gallic acid, kaempferol, myricetin, neochlorogenic acid, procyanidin dimer B2, quercetin, rutin, and sinapic acid as well as bibliographic information. Compounds were numbered by their retention time, keeping the same numbering in all samples. A brief explanation of the characterization follows.

Flavonoids

Most of the flavonoids found in the extracts were glycosylated flavonoids. They were characterized by the neutral losses of 308 Da (rutinoside), 176 Da (glucuronide), 162 Da (hexoside), 152 Da (gallate), 146 Da (deoxyhexoside) and 132 Da (pentoside). Aglycones were identified by comparison with analytical standards or bibliographic information.

Catechin (compound **17**) was identified by comparison with an analytical standard. Compound **28**, with the same fragmentation pattern, was thus identified as epicatechin. Several compounds were characterized as (epi)catechin derivatives. Compounds **9** and **12** were characterized as (epi)gallocatechin isomers, whereas compound **30** presented a fragmentation pattern consistent with (epi)gallocatechin gallate (Stewart et al., 2005). Compounds **11**, **19**, **21**, **22**, **23**, **25** and **27** were characterized as proanthocyanidin dimers and trimers (Hamed et al., 2014; Kajdžanoska et al., 2010). Compounds **34**, **38**, **41**, **43**, **47** and **49** were myricetin glycosides. The aglycone myricetin was observed at *m/z* 317 (main fragment ions at *m/z* 271, 179 and 151). Compounds **45**, **48**, **50**, **51**, **52**, **61**, **66**, **70**, **72** and **77** were characterized as quercetin glycosides, depicting quercetin aglycone at *m/*z 301 (fragment ions at *m/z* 179 and 151). Compounds **29**, **53**, **62**, **73** and **74** were kaempferol glycosides. Kaempferol was observed in all cases at m/z 285 (comparison with an analytical standard). Compound **54**, with deprotonated molecular ion at *m/z* 493, suffered the neutral loss of 162 Da to yield mearnsetin at *m/z* 331 (main fragment ion at *m/z* 316) (Han et al., 2008). It was thus characterized as mearnsetin-*O*-hexoside. Compound **65** suffered the neutral loss of 80 Da (sulfate) to yield isorhamnetin at *m/z* 315 (main fragment at *m/z* 300). Compound **56** was identified as

isorhamnetin-*O*-hexoside due to the loss of 162 Da to yield isorhamnetin at *m/z* 315. Compound **68** suffered the loss of 162 Da to yield apigenin at m/z 269 (comparison with an analytical standard), so it was characterized as apigenin-O-hexoside. Compounds **35** and **42**, with $[M-H]^-$ at *m/z* 563, were characterized as apigenin-*C*-hexoside-*C*-pentoside isomers due to the fragment ions observed at [M-H-60]-, [M-H-90]-, [M-H-120]-, [M-H-180]-, and [M-H-210]-, characteristic of di-*C*-glycoside flavonoids (Han et al., 2008). Compound **79** was identified as luteolin by comparison with an analytical standard. Compounds **31**, **37**, **40** and **57** were luteolin-*C*-glycosides. Compound **37** was specifically identified as isoorientin due to the fragment ion at *m/z* 429 (absent in orientin; Algamdi et al., 2011). Compounds **67**, **71**, **76** and **80** exhibited similar fragmentation patterns: losses of 80 Da (sulfate) and 15 Da (methyl groups). They were tentatively characterized as methylated flavonoids, as they presented maximum UV wavelengths at approximately 350 nm, typical of flavonoids.

Phenolic acids

Compounds **3** and **8** displayed the neutral loss of 162 Da (hexoside) to yield the base peak at *m/z* 153, which was characterized as a dihydroxybenzoic acid (comparison with a protocatechuic acid analytical standard). Several caffeoylquinic acids (compounds **4**, **10** and **18**) and dicaffeoylquinic acids (compounds **44**, **59**, **63** and **69**) were characterized by using analytical standards and bibliographic information (Clifford et al., 2005). Compounds **16**, **24** and **26** were identified as hydroxycinnamic acids - sinapic acid, ferulic acid and caffeic acid, respectively – by comparison with analytical standards. For the characterization of compound **58**, we used the positive ion mode. The protonated molecular ion and fragmentation pattern were consistent with hydroferuloylglucose according to bibliographic data (Ma et al., 2007).

Others

Compound **1** suffered the neutral loss of 36 Da (HCl) to yield the base peak at *m/z* 341. Its fragmentation pattern was consistent with a disaccharide formed by two hexosides (probably glucose) (Brudzynski et al., 2011). Compound **2**, with deprotonated molecular ion at *m/z* 191, presented fragment ions at *m/z* 173 and 111. Although citric acid presents a similar fragmentation pattern, this compound was characterized as quinic acid after the analysis of a citric acid analytical standard (which showed a different

retention time). Compound **7** was identified as gallic acid due to the $[M-H]^-$ at *m/z* 169 and base peak at *m/z* 125. Several derivatives were also tentatively characterized. Compounds **6** presented fragment ions at *m/z* 191 (quinic acid) and 169/125 (gallic acid), so it was characterized as galloylquinic acid; with an additional 152 Da (gallic moiety), compounds **5**, **13** and **14** were characterized as di-*O*-galloylquinic acid isomers (Bastos et al., 2020). Compound **15** was characterized as methyl gallate (Li et al., 2016) and **32** as a gallic acid derivative. Compound **64** was identified as pinoresinol by comparison of its experimental mass spectra with bibliographic data (Ye et al., 2005). Compounds **81** and **82** were tentatively characterized as the lignans oxo-dihydroxy-octadecenoic and trihydroxy-octadecenoic acids (Van Hoyweghen et al., 2014).

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Table SI. Quantification of the main compounds detected in *Pistacia lentiscus* extract, in non-treated (-) and treated-PVPP (+) samples.

Nº	**Assigned identification**	**mg g^{-1} DW extract**	
		PVPP (-)	**PVPP (+)**
Flavonoids			
9	(Epi)gallocatechin	6.4 ± 0.4	--
17	Catechin	5.0 ± 0.3	--
30	Gallocatechin gallate	6.8 ± 0.4	--
34	Myricetin-Hex-gallate	0.14 ± 0.01	--
38+41+43	Myricetin glycosides	21 ± 1[a]	0.17 ± 0.01[b]
45	Quercetin-Hex-gallate	0.26 ± 0.02	--
47+49	Myricetin glycosides	19 ± 1	--
51	Quercetin-*O*-Hex	3.5 ± 0.2	--
61+62	Quercetin-Pen + Kaempferol-Hex	2.2 ± 0.1	--
66	Quercetin-*O*-dHex	6.0 ± 0.4	--
70	Quercetin-Pen-gallate	0.76 ± 0.05	--
73	Kaempferol-*O*-dHex	0.59 ± 0.04	--
74	Kaempferol-Pen-gallate	0.24 ± 0.02	--
79	Luteolin	1.13 ± 0.06	--
Total		**73 ± 2[a]**	**0.17 ± 0.01[b]**
Others			
6	Galloylquinic acid	15 ± 1[a]	2.2 ± 0.2[b]
13	di-O-Galloylquinic acid	21 ± 1	--
14	di-O-Galloylquinic acid	8.3 ± 0.5	--
15	Methyl gallate	16 ± 1	--
Total		**60 ± 2[a]**	**2.2 ± 0.2[b]**
TIPC		**133 ± 3[a]**	**2.4 ± 0.2[b]**

Bold values represent the sum of each type of components. Different superscripts in the same line mean significant differences.

Table SII. Quantification of the main compounds detected in *Helichrysum italicum picardii* extract, in non-treated (-) and treated-PVPP (+) samples.

Nº	**Assigned identification**	**mg g^{-1} DW extract**	
		PVPP (-)	**PVPP (+)**
Phenolic acids			
8	Dihydroxybenzoic acid-*O*- Hex	0.27 ± 0.03[a]	0.21 ± 0.02[a]
10	Neochlorogenic acid	2.6 ± 0.2[a]	0.99 ± 0.07[b]
18	Chlorogenic acid	14.1 ± 0.7[a]	6.3 ± 0.4[b]
44	Dicaffeoylquinic acid	3.5 ± 0.2	--
59	Dicaffeoylquinic acid	6.4 ± 0.3	--
63	Dicaffeoylquinic acid	93 ± 4[a]	0.51 ± 0.03[b]
69	Dicaffeoylquinic acid	30 ± 2[a]	0.33 ± 0.02[b]
Total		**150 ± 5[a]**	**8.3 ± 0.4[b]**
Flavonoids			
50	Quercetin-*O*-Hex	19 ± 1	--
54	Mearnsetin-*O*-Hex	5.7 ± 0.4	--
56	Isorhamnetin-*O*-Hex	1.7 ± 0.1	--
61	Quercetin-*O*-Pen	8.1 ± 0.4	--
72	Quercetin-*O*-Hex	1.6 ± 0.1	--
77	Quercetin-*O*-dHex-*O*-Hex	1.9 ± 0.1	--
Total		**38 ± 1**	**--**
TIPC		**188 ± 5[a]**	**8.3 ± 0.4[b]**

Bold values represent the sum of each type of components. Different superscripts in the same line mean significant differences.

Table SIII. Quantification of the main compounds detected in *Cladium mariscus* extract in non-treated (-) and treated-PVPP (+) samples.

Nº	**Assigned identification**	**mg g^{-1} DW extract**	
		PVPP (-)	**PVPP (+)**
Catechin derivatives			
9	(Epi)gallocatechin	0.47 ± 0.03	--
11+12	Proanthocyanidin dimer+(epi)gallocatechin	4.6 ± 0.3	--
Total		**5.1 ± 0.3**	**--**
Flavonoids			--
31	Luteolin-*C*-Hex-*C*-Pen	2.0 ± 0.1[a]	0.70 ± 0.05[b]
35	Apigenin-*C*-Hex-*C*-Pen	0.49 ± 0.03[a]	0.37 ± 0.03[b]
37	Luteolin-6-*C*-glucoside (isoorientin)	2.0 ± 0.1	--
53	Kaempferol-*O*-Hex	0.97 ± 0.06	--
57	Luteolin-*C*-Pen	1.0 ± 0.07	--
79	Luteolin	3.0 ± 0.2	--
Total		**9.5 ± 0.3[a]**	**1.07 ± 0.06[b]**
TIPC		**14.6 ± 0.4[a]**	**1.07 ± 0.06[b]**

Bold values represent the sum of each type of components. Different superscripts in the same line mean significant differences.

Table SIV. Quantification of the main compounds detected in *Limoniastrum monopetalum* extract, in non-treated (-) and treated-PVPP (+) samples.

Nº	**Assigned identification**	**mg g^{-1} DW extract**	
		PVPP (-)	**PVPP (+)**
Phenolic acids			
7	Gallic acid	3.8 ± 0.2	--
16	Sinapic acid sulfate	0.37 ± 0.03[a]	0.33 ± 0.02[a]
24	Ferulic acid sulfate	0.83 ± 0.05[a]	0.46 ± 0.03[b]
32	Gallic acid derivative	5.3 ± 0.3[a]	0.65 ± 0.04[b]
Total		**10.3 ± 0.4[a]**	**1.44 ± 0.05[b]**
Flavonoids			
9	(Epi)gallocatechin	8.6 ± 0.4	--
12	(Epi)gallocatechin	0.86 ± 0.05	--
65	Isorhamnetin sulfate	6.4 ± 0.3	--
Total		**15.9 ± 0.5**	**--**
TIPC		**26.2 ± 0.6[a]**	**1.44 ± 0.05[b]**

Bold values represent the sum of each type of components. Different superscripts in the same line mean significant differences.

CHAPTER IV

IMPACT OF SEASONAL AND ORGAN-RELATED FLUCTUATIONS ON THE ANTHELMINTIC PROPERTIES AND CHEMICAL PROFILE OF *CLADIUM MARISCUS* (L.) POHL EXTRACTS

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Impact of seasonal and organ-related fluctuations on the anthelmintic properties and chemical profile of *Cladium mariscus* (l.) Pohl extracts

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ABSTRACT

The use of plants and their metabolites stands as a promising option to tackle parasitic infections by gastrointestinal nematodes (GIN) in integrated control strategies. Still, the influence of environmental and phenological factors, and their interactions, in the wild on the metabolomics and biological properties of target plant species, is often disregarded. In this work, we hypothesized that variations in the anthelmintic (AH) properties and chemical composition of extracts from the salt tolerant species *Cladium mariscus* L. Pohl (sawgrass) may be influenced by seasonal factors and organ-parts. To test this hypothesis, acetone/water extracts were prepared from dried biomass obtained from aerial organs collected from sawgrass in consecutive seasons and tested against *Haemonchus contortus* and *Trichostrongylus colubriformis* by the larval exsheathment inhibition assay (LEIA) and egg hatching inhibition assay (EHIA). To ascertain the role of plant organ, the activity of leaves and inflorescences extracts from summer samples was compared. The role of polyphenols in the anthelmintic activity depending on GINs and fluctuations across seasons and plant organs was assessed using polyvinylpolypyrrolidone (PVPP), coupled with an in-depth chemical profiling analysis using high-performance liquid chromatography completed with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n). Main differences in anthelmintic activities were observed for summer and autumn samples, for both assays. Moreover, inflorescences' extracts were significantly more active than those from leaves against both parasite species on EHIA and against *H. contortus* on LEIA. Application of PVPP totally inhibits the AH effects based on EHIA and only partly for LEIA. Non-treated PVPP extracts were predominantly composed of flavan-3-ols, proanthocyanidins, luteolin and glycosylated flavonoids, while two flavonoid glycosides were quantified in all PVPP-treated samples. Thus, the activity of such compounds should be further explored, although some unknown metabolites remain to be identified. This study reinforces the hypothesis of the AH potential of sawgrass and of its polyphenolic metabolites uses as nutraceutical and/or phytotherapeutic drugs.

Keywords: halophytes, salt tolerant plants, anthelmintic, polyphenols, gastrointestinal nematodes, small ruminants

4.1. INTRODUCTION

Parasitic infections by gastrointestinal nematodes (GIN) represent a serious economical and health threat to outdoor production systems of small ruminant worldwide, where *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., and *Nematodirus* spp. are the most prevalent species (Charlier et al., 2018). As increasing anthelmintic resistances are reported, mainly due to the indiscriminate administration of available commercial synthetic drugs (Rose Vineer et al., 2020), it becomes peremptory to find novel GIN integrated control options. A promising suggested approach is the use of plants and their bioactive products, either as nutraceuticals, phytotherapeutic remedies, including essential oils as sources of secondary metabolites of veterinary interest to control parasites (Hoste et al., 2015).

Increasing evidence of *in vitro* and *in vivo* anthelmintic effects has been described for a range of botanical species and linked to a variety of bioactive metabolites, with particular emphasis on plants rich in polyphenolic compounds, specifically tannins (Hoste et al., 2006, 2015; Manolaraki, 2011; Spiegler et al., 2017; Santos et al., 2019; Liu et al., 2020). Such plants include both glycophytes, for example Legume forages (e.g., sainfoin: *Onobrychis viciifolia* Scop. and sulla: *Hedysarum coronarium* L.) and halophytes [e.g., mastic tree: *Pistacia lentiscus* L. and sawgrass: *Cladium mariscus* (L.) Pohl] (Oliveira et al., 2021c). Halophytes are high salt tolerant plants able to cope with several abiotic stressors, besides salinity, for example, high light intensity, high UV radiation, and drought/flood. That capacity is possible due to several adaptation mechanisms, including the synthesis of high levels of antioxidant secondary molecules, such as polyphenols. Polyphenols are therefore produced as part of the plant defense machinery, and as such, the extent of its production is influenced by multiple complex abiotic and biotic interactions. Several studies on different models of bioactive plants rich in polyphenols have shown that the concentration of these compounds and, consequently, their pharmacological properties, rely to a range of factors either botanical (*e.g.*, plant species, organs; plant cultivars and varieties, phenological stages), geographical and environmental (e.g., season, area of collection), as well as cultivation systems (Manolaraki, 2011). Similar factors have been described for halophytes wild plants. For example, high light intensity, UV irradiance, and saline stress enhanced the biosynthesis of antioxidant flavonoids in *Ligustrum vulgare* L., particularly quercetin and luteolin

glycosides (Tattini et al., 2004; Agati et al., 2011). Variations on the chemical composition and bioactive properties of plant organs (*e.g.,* leaves, stems, flowers) are described for different salt-tolerant species such as *Eryngium maritimum* L., *Limoniastrum monopetalum* L., and *Limonium algarvense* L. (Trabelsi et al., 2012; Rodrigues et al., 2015; Pereira et al., 2019). In addition, Azaizeh et al. (2013, 2015) observed that the polyphenol content and anthelmintic effects of *P. lentiscus* L. and *Inula viscosa* L. extracts on third stage (L3) mixed-species larvae exsheathment process changes across seasons. Thus, seasonality and organ-related variations should be taken into consideration when evaluating the anthelmintic value of botanical species aiming at its future use for GIN control strategies.

Cladium mariscus (L.) Pohl (Cyperaceae), also known as sawgrass, is a grass-like perennial halophytic herbaceous species distributed along the Mediterranean region, in low to moderate saline environments (Gerdol et al., 2018). Sawgrass lengthy and long-lasting leaves have characteristic saw-shaped margins and inflorescences rise above leaves (Castroviejo, 2008; Figure 4.1). In our previous investigations, sawgrass 80% acetone/water extracts exhibited high polyphenol content coupled with *in vitro* antioxidant and anti-inflammatory properties, with marked differences among seasons (Lopes et al., 2016; Oliveira et al., 2021a). Moreover, sawgrass aerial organs extract suppressed egg hatching and L3 larvae exsheathment processes of two models of GINs of sheep and goats, namely one abomasal species *Haemonchus contortus* and one intestinal species *Trichostrongylus colubriformis* (Oliveira et al., 2021b).

In this work, we hypothesize that (1) differences in anthelmintic effects can occur among seasons and between aerial organs of sawgrass, and that (2) polyphenols could have a role on the detected anthelmintic effects. The first hypothesis was explored by using sawgrass extracts prepared from biomass collected from wild plants during the four seasons, and from different plant organs, namely leaves and inflorescences. Extracts were *in vitro* tested relying on two nematode stages (egg hatching and L3 larvae), and two key nematode species (*H. contortus* and *T. colubriformis*). To test the second hypothesis, polyvinylpolypyrrolidone (PVPP), a polyphenol-binding agent, was added to the extracts and the metabolites in non-treated and treated samples were profiled by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MS^n).

4.2. MATERIAL AND METHODS

4.2.1. Plant collection and processing

Cladium mariscus (L.) Pohl aerial parts, including leaves and inflorescences (Figure 4.1), were manually harvested in Ludo, Faro, southern Portugal (37º01'03.3" N, 7º59'18.1"W, aprox. 7 m elevation) in spring (Sp; April, 2017: minimum temperature: 12 ºC, maximum temperature: 21 ºC, mean precipitation: 34 mm), summer (Su; July 2017: minimum temperature: 19 ºC, maximum temperature: 29 ºC; mean precipitation: 1 mm), autumn (Au; October, 2017: minimum temperature: 14 ºC, maximum temperature: 25 ºC, mean precipitation: 55 mm) and winter (Wi; January, 2018: minimum temperature: 8 ºC, maximum temperature: 16 ºC; mean precipitation: 210 mm) (source: WeatherSpark based on data obtained in the Faro International Airport). Inflorescences were present during summer and, to a lesser extent, in autumn, while green leaves were collected all year. In addition, only for summer season, samples of leaves (Le) and inflorescences (Inf) were also collected, for separate analysis. Afterward, samples were transported to the laboratory, washed, frozen at -20 ºC until freeze-dried (Lyoalfa 15) for 3 days and reduced to powder using a coffee and a ball miller (Retsch PM 100). For plant collection in the wild, mandatory licenses for the Portuguese territory were obtained, and the protocol for collection was followed according to the standard procedures recommended by "*Instituto da Conservação da Natureza e das Florestas* (ICNF)", the national regulatory body. Voucher specimen (XBH03) were kept in the XtremeBio group herbarium, at Centre of Marine Sciences (CCMAR), University of Algarve (UAlg), Faro, Portugal, and the formal identification of the botanical material was made by Dr. Luísa Custódio (CCMAR).

4.2.2. Sample preparation

Dried biomass was extracted with an 80% aqueous acetone solution (1:40, w/v) at room temperature (20–25 ºC), for 16 h, under stirring. Then, the residue was sieved using qualitative filters and concentrated in a rotary evaporator under reduced pressure and temperature (approximately 40 ºC), aiming acetone removal. Afterward, the aqueous residue was freeze-dried, and the extracts recovered for use in further anthelmintic and chemical assays.

Figure 4.1 Illustration of *Cladium mariscus* (L.) Pohl (sawgrass) aerial organs: inflorescences (**A**) and detail of leaves (**B**). Illustration by M. Oliveira.

4.2.3. *In vitro* anthelmintic assays

4.2.3.1. Haemonchus contortus and Trichostrongylus colubriformis parasites

The feces of caprine and ovine donors, kept indoors, monospecifically infected with susceptible strains of either *H. contortus* or *T. colubriformis*, were used to obtain third stage larvae (L3) and eggs. The facilities hosting the animals and trial performance met French ethical and welfare rules (agreement C 31 555 27 of August 19, 2010). Larvae were recovered with the Baermann method, stored in culture flasks at 4 ºC for one (*H. contortus*) or 4 months (*T. colubriformis*), before use in the larval exsheathment inhibition (LEIA) experiments. Eggs were collected on the day of the egg hatching inhibition (EHIA) experiments and used up to 2 h prior to collection.

4.2.3.2. Larval Exsheathment Inhibition assay (LEIA)

Larval exsheathment inhibition assay (LEIA) protocol was performed as previously described by Bahuaud et al. (2006). The extracts were prepared in serial concentrations of 1,200, 600, 300, 150, and 75 µg mL^{-1} in phosphate buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH 7.2) and incubated at 23 ºC for 3 h with L3 larvae (approx. 800 larvae mL^{-1}). After three times washed and centrifuged, the pellet was resuspended in 200 µL of PBS, and 40 µL were used to count the proportion of ensheathed/exsheathed larvae at 0 min. Then, the remaining 160 µL were exposed to a solution of Milton (2% w/v sodium hypochlorite, 16.5% w/v sodium chloride in PBS), aiming at the artificial induction of larvae exsheathment. The optimal concentration of Milton's solution to achieve a gradual exsheathment process, i.e., reaching 100% exsheathment in 60 min, was tested for each batch before use. After 20, 40, and 60 min of exposure, the number of ensheathed and exsheathed larvae were counted under a microscope (400×). For each extract concentration and negative control (PBS) four replicates were performed and run in parallel. Percentage of larvae exsheathment (LE) for each replicate, was given by the formula: %LE = [(number of exsheathed larvae)/(number of exsheathed + ensheathed larvae) × 100].

4.2.3.3. Egg Hatching Inhibition Assay (EHIA)

Egg hatching inhibition assay (EHIA) protocol was performed as previously described in Oliveira et al. (2021b). Fecal samples were filtrated twice using a gaze hydrophile compress, sieved (25 mm) and washed with distilled water. The residues were subjected to three centrifugation steps using a saline saturated solution (d = 1.2), and the

pellet recovered in PBS. After quantification, eggs (100 per well) were plated in 48-well sterile plates and treated with the extract solutions, prepared in PBS in concentrations ranging from 5,000 to 78 μg mL^{-1}. After a 48-h incubation period at 27 ºC, the number of larvae and eggs, in each well, was registered after microscopic counting. Six replicates were performed for each extract concentration and the negative control, PBS, was run in parallel. The percentage of egg hatching (EH) for each well, was obtained as given by the following formula: %EH = [(number of larvae)/(number of eggs + larvae) × 100].

4.2.4. Polyvinylpolypyrrolidone treatment

To appraise the possible role of polyphenols in the anthelmintic activity, PVPP, a polyphenol inhibitor (Doner et al., 1993) was added to extracts solutions (ratio 50:1; w/w) at the concentrations of 2,500 μg mL^{-1} and 1,200 μg mL^{-1}, as previously described by Oliveira et al. (2021b). After overnight incubation at 4 ºC, the solution was centrifuged for 10 min at 4,500 rpm and the supernatant posteriorly used in the *in vitro* assays and for chemical analysis. The extracts not exposed to PVPP and the negative control, PBS, were run in parallel.

4.2.5. Chemical profiling by High-performance Liquid chromatography with Electrospray Ionization Mass Spectrometric detection

Chromatographic analyses were performed with an Agilent Series 1100 HPLC system with a G1315B diode array detector (Agilent Technologies, Santa Clara, CA, United States) and an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics, Billerica, MA, United States) with an electrospray interface. Separation was performed in a Luna Omega Polar C18 analytical column (150 × 3.0 mm; 5 mm particle size) with a Polar C18 Security Guard cartridge (4 × 3.0 mm), both purchased from Phenomenex, Torrance, CA, United States. Detailed chromatographic conditions are available in Fernández-Poyatos et al. (2019). Compounds' identification was performed by mass spectrometry data, whereas the quantification was carried out by UV using analytical standards of catechin (280 nm), apigenin (350 nm), kaempferol (350 nm) and luteolin (350 nm). Calibration graphs were constructed in the 0.5–100 mg mL^{-1} range. Repeatability (n = 9) and intermediate precision (n = 9, 3 consecutive days) were lower than 3 and 8%, respectively. Each analytical standard was used to quantify the corresponding compound or compounds of the same chemical family. The characterization of the metabolites was carried out by HPLC-ESI-MS^n using the negative

ion mode and identification was performed using analytical standards as well as bibliographic information. Compounds were numbered according to their elution order, keeping the same numbering in all extracts.

4.2.6. Statistical analysis

Results are expressed either as effective concentration that inhibits 50% of the larval exsheathment and/or egg hatching (EC_{50}) values (μg mL^{-1}) including 95% confidence intervals (CI). EC_{50} values and 95% CI were obtained by Probit analysis, while significant differences among groups were detected by relative median potency estimates, through IBM SPSS Statistics v. 26.0 software. PVPP results are expressed as average ± standard error of the mean (SEM).

4.3. RESULTS

4.3.1. Season- and organ-related effects on *in vitro* anthelmintic properties

Table 4.1 summarizes the *in vitro* anthelmintic activity results of sawgrass extracts prepared from biomass collected in successive seasons, against *H. contortus* and *T. colubriformis* larvae and eggs. Concerning the LEIA results, no significant seasonal effects were recorded for *H. contortus*, however, the summer sample was the most effective on inhibiting *T. colubriformis* L3 larvae exsheathment (EC_{50} = 77.8 µg mL^{-1}), followed by the autumn extract (EC_{50} = 110.9 µg mL^{-1}). Spring and winter samples were less active on *T. colubriformis* than *H. contortus*, while summer and autumn samples inhibited larvae exsheathment from both species in a similar manner. The extracts consistently presented lower EC_{50} values toward L3 larvae exsheathment than egg hatching processes and were more effective on impairing egg hatching of *H. contortus* than those of *T. colubriformis* (Table 4.1). While no seasonal effects were observed on EHIA using *T. colubriformis* parasites, the summer extract was the most active against *H. contortus* (EC_{50} = 1446.6 µg mL^{-1}). The inflorescence extract was significantly more active to inhibit larvae exsheathment of *H. contortus* (EC_{50} = 60.0 µg mL^{-1}) than leaves (EC_{50} = 87.7 µg mL^{-1}). Indeed, the inflorescences extracts were also more effective than leaves against egg hatching, as illustrated by EC_{50} values for inflorescence representing 37% of those obtained for leaves, for both parasite species (Table 4.2). Interestingly, despite the high activity of inflorescences sample on EHIA, this is not reflected in the seasonal samples, particularly in summer as marked differences are not observed between samples collected in different seasons.

Table 4.1 Effective concentration that inhibits 50% of larval exsheathment or egg hatching (EC_{50} values, µg mL^{-1}) and 95% confidence intervals (CI) obtained for *Cladium mariscus* (L.) Pohl (sawgrass) extracts on *Haemonchus contortus* and *Trichostrongylus colubriformis* L3 larvae exsheathment (LEIA) and egg hatching inhibition assays (EHIA).

	LEIA		EHIA	
	H. contortus	***T. colubriformis***	***H. contortus***	***T. colubriformis***
Spring	94.0^{Aa} (72.3–123.4)	159.6^{Bb} (125.7–211.3)	1902.8^{Ab} (1712.9–2126.3)	2275.3^{Ba} (2028.9–2564.6)
Summer	88.9^{Aa} (66.3–118.7)†	77.8^{Aa} (60.6–100.0)†	1496.6^{Aa} (1326.5–1698.9)†	2575.5^{Ba} (2324.1–2881.8)†
Autumn	99.6^{Aa} (77.8–128.3)	110.9^{Aab} (88.4–140.8)	1826.3^{Ab} (1622.9–2069.8)	2384.6^{Ba} (2118.3–2704.7)
Winter	70.4^{Aa} (52.2–97.0)	128.9^{Bb} (97.2–176.3)	1873.2^{Ab} (1688.5–2087.1)	2386.3^{Ba} (2137.5–2681.7)

†Oliveira et al., 2021b. For each assay, different letters represent significant statistical differences ($p < 0.05$) either between GIN species (capital letter within rows) and/or between seasons (small letters; within columns), respectively, based on Relative Median Potency Estimates.

Table 4.2 Effective concentration that inhibits 50% of larval exsheathment or egg hatching (EC_{50} values, μg mL^{-1}) and 95% confidence intervals (CI) obtained for *Cladium mariscus* (L.) Pohl (sawgrass) organ extracts on *Haemonchus contortus* and *Trichostrongylus colubriformis* L3 larvae exsheathment (LEIA) and egg hatching inhibition assays (EHIA).

	LEIA		EHIA	
	H. contortus	***T. colubriformis***	***H. contortus***	***T. colubriformis***
Leaves	87.7^{Ab} (71.3–108.5)	81.1^{Aa} (67.2–98.5)	2079.4^{Ab} (1927.8–2248.1)	2289.9^{Ab} (2118.7–2481.3)
Inflorescences	60.0^{Aa} (47.6–74.9)	78.6^{Aa} (64.6–96.2)	776.5^{Aa} (732.3–824.7)	848.2^{Aa} (797.6–903.5)

Different letters represent significant statistical differences among GIN species (capital; rows) and plant organs (small; columns) for each assay, respectively, based on Relative Median Potency Estimates.

4.3.2. Role of polyphenols in the anthelmintic activity: polyvinylpolypyrrolidone as a polyphenol binding agent

All PVPP-treated extracts, including those from leaves and inflorescences, still exhibit activity against the *H. contortus* and *T. colubriformis* L3 exsheathment in a similar manner (10–40% larvae exsheathment), while egg hatching was completely restored (Figures 4.2–4.4).

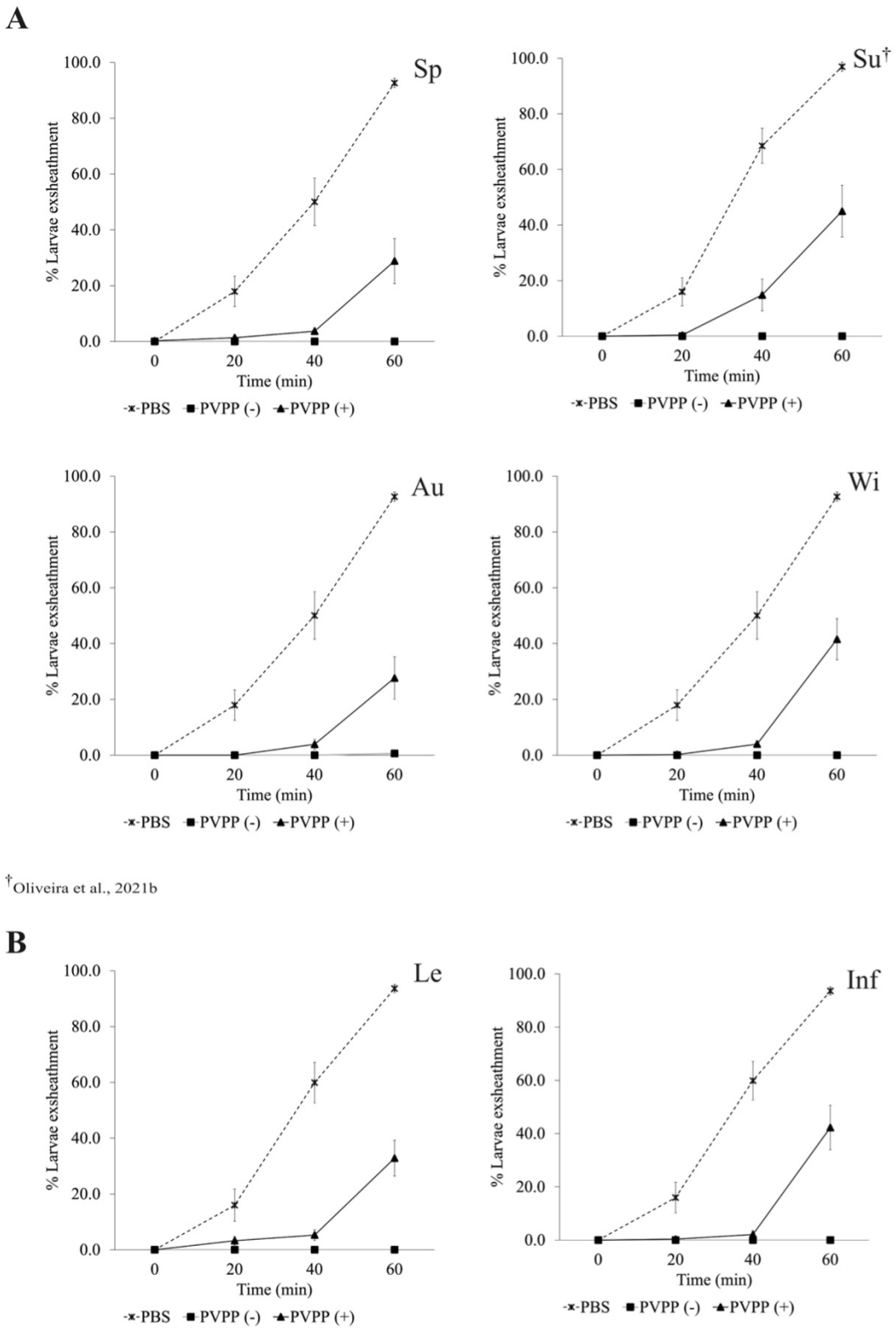

Figure 4.2 Anthelmintic effects of *Cladium mariscus* (L.) Pohl (sawgrass) seasonal (**A**) and organ (**B**) extracts, on LEIA for *Haemonchus contortus* at the concentration of 1200 μg mL^{-1}, either treated [PVPP(+)] or not [PVPP(−)] with PVPP. **Sp**, Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences.

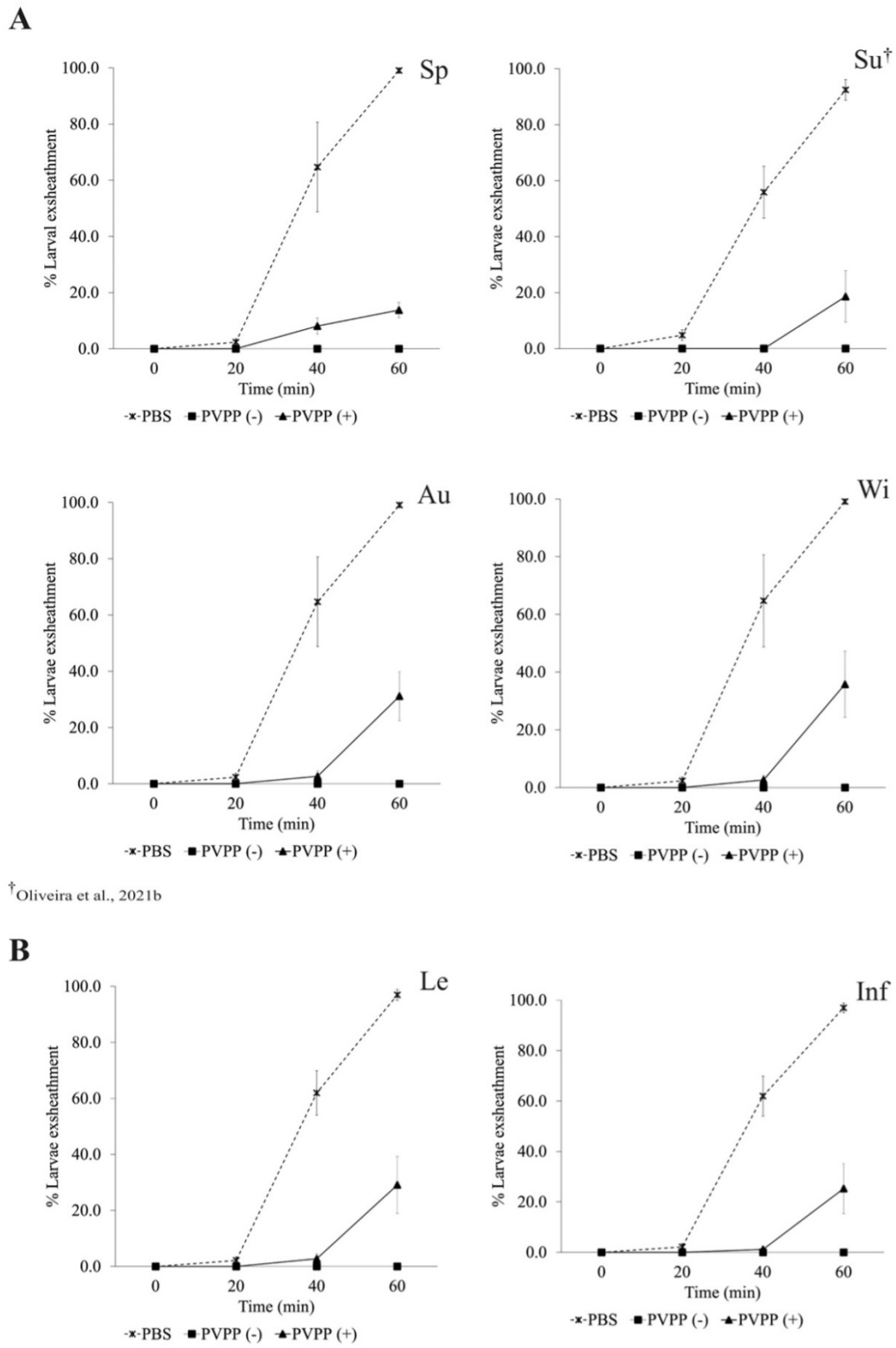

Figure 4.3 Anthelmintic effects of *Cladium mariscus* (L.) Pohl (sawgrass) seasonal (**A**) and organ (**B**) extracts, on LEIA for *Trichostrongylus colubriformis* at the concentration of 1200 µg mL^{-1}, either treated [PVPP(+)] or not [PVPP(−)] with PVPP. **Sp**, Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences.

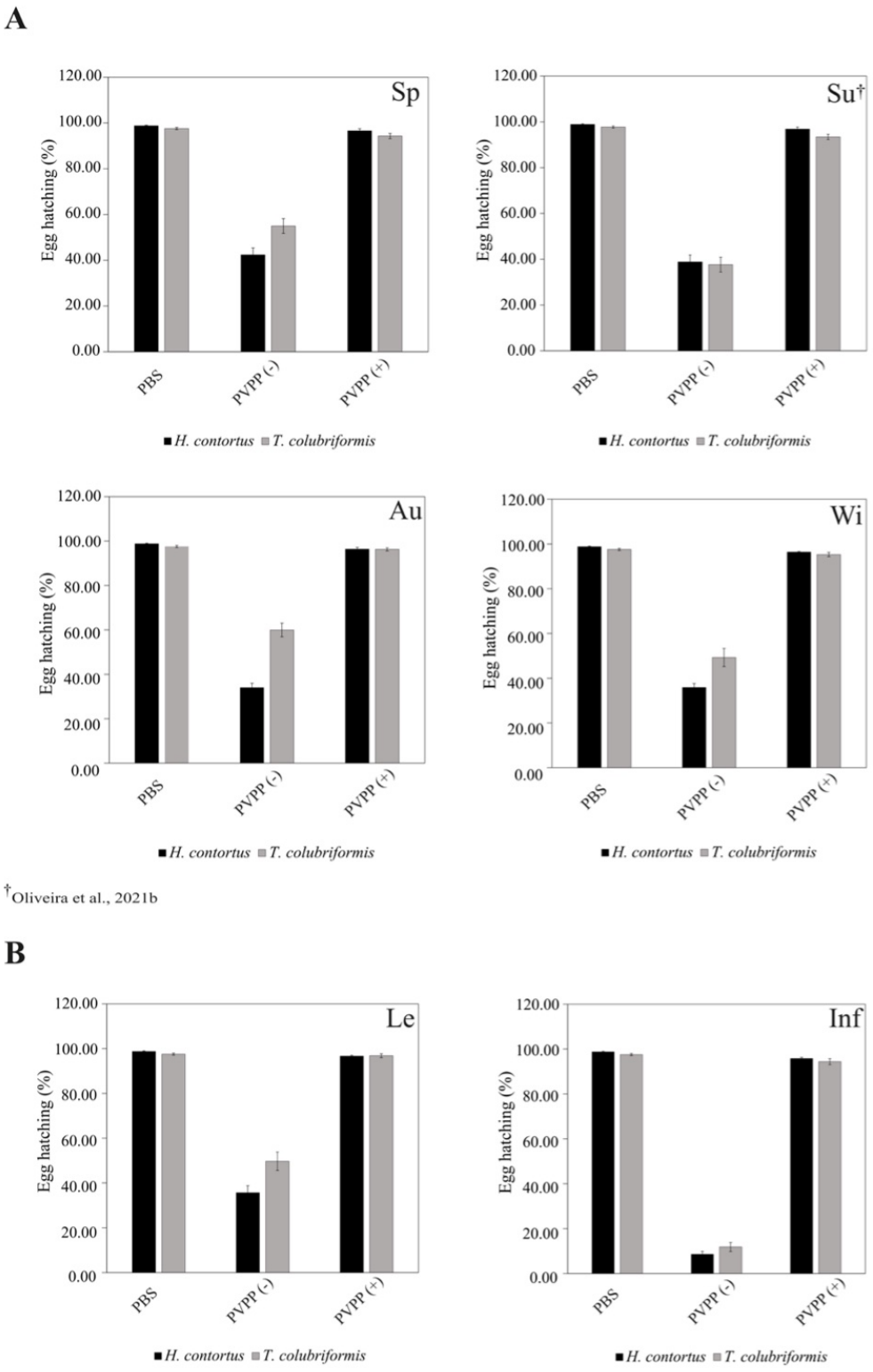

Figure 4.4 Anthelmintic effects of *Cladium mariscus* (L.) Pohl (sawgrass) seasonal (**A**) and organ (**B**) extracts on EHIA for *Haemonchus contortus* and *Trichostrongylus colubriformis* at the concentration of 2500 μg mL^{-1}, either treated [PVPP(+)] or not [PVPP(−)] with PVPP. **Sp**, Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences.

4.3.3. High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric detection comparative analysis of the chemical profile of Polyvinylpolypyrrolidone treated vs. non-treated extracts

The chemical characterization of the metabolites in each extract carried out by HPLC-ESI-MS^n analysis is depicted in Table 4.3, while quantification of the main compounds of non-treated and treated PVPP samples is presented in Tables 4.4, 4.5. Compounds were numbered according to their elution order, keeping the same numbering in all extracts.

All extracts were mainly composed of flavonoids, specifically flavan-3-ols (epigallocatechin, epicatechin 14) and proanthocyanidins (condensed tannins; 2.3–35.3 mg g^{-1}, DW), luteolin (0.31–6.1 mg g^{-1}, DW) and glycosylated flavonoids (1.9–27.6 mg g^{-1} DW; Table 4.4). Summer and autumn samples had the highest amounts of epicatechin 14 (3.4–5.3 mg g^{-1}, DW) and of luteolin 29 (3.5–4.1 mg g^{-1}, DW). Compound **5** was identified as catechin by comparison with an analytical standard. Compound 14, with the same fragmentation pattern, was thus identified as epicatechin. Several compounds were characterized as (epi)catechin derivatives. Compounds **2** and **4** were characterized as (epi)gallocatechin isomers. Compounds **3**, **7**, and **9–13** were characterized as proanthocyanidin dimers and trimers (Kajdžanoska et al., 2010; Hamed et al., 2014).

Compounds **16** and **20**, with [M-H]- at m/z 563, were characterized as apigenin-C-hexoside-C-pentoside isomers due to the fragment ions observed at [M-H- 60]-, [M-H- 90]-, [M-H- 120]-, [M-H- 180]-, and [M-H- 210]-, characteristic of di-C-glycoside flavonoids (Han et al., 2008). Compound **22** was characterized as apigenin-8-C-glucoside (vitexin) based on bibliographic information (Waridel et al., 2001). Compound **23** was characterized as kaempferol-*O*-hexoside due to the neutral loss of 162 Da to yield kaempferol aglycone at m/z 285. The fragmentation of kaempferol was compared with an analytical standard. Compound **27** was identified as myricetin, with deprotonated molecular ions at m/z 317 and fragment ions at m/z 179 and 151. Compound **21** was characterized as myricetin-*O*-deoxyhexoside. Compound **29** was identified as luteolin by comparison with an analytical standard. Compounds **15**, **17**, **19**, and **24** were luteolin-C-glycosides. Compound **17** was characterized as isoorientin due to the fragment ion at m/z 429 (absent in orientin) (Algamdi et al., 2011). Besides flavonoids, one phenolic acid, compound **6,** was identified as chlorogenic acid by comparison with an analytical standard. Moreover, compound **1** suffered the neutral loss of 36 Da (HCl) to yield the

base peak at m/z 341. Its fragmentation pattern was consistent with a disaccharide formed by two hexosides (probably glucose) (Brudzynski and Miotto, 2011).

The inflorescences extract (from the summer sample) exhibited increased amounts of compounds **14** and **29** (6.1 and 6.1 mg g^{-1} DW), in contrast to leaves (0.17 and 0.78 mg g^{-1} DW). The proanthocyanidin dimer + (epi)gallocatechin **3** + **4** reached a maximum of 30 mg g^{-1}, dry weight (DW) in autumn. High amounts of **3 + 4** are noted in all extracts ranging from 2.1 up to 30 mg g^{-1} DW, except for inflorescences (0.84 mg g^{-1} DW). Proanthocyanidins **7** and **9–13** were identified but not quantified due to low signal.

Table 4.3 Characterization of the polyphenolic compounds found in the analyzed extracts of *Cladium mariscus* (L.) Pohl (sawgrass). **Sp**, Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences.

No.	t_R (min)	$[M-H]^-$ *m/z*	m/z (% base peak)	Assigned identification	PVPP (-)	PVPP (+)
1	1.8	377	MS^2 [377]: 341 (100) MS^3 [377→341]: 179 (100), 161 (24), 143 (13), 119 (25), 113 (20)	Disaccharide (HCl adduct)	All samples	All samples
2	4.6	305	MS^2 [305]: 261 (7), 221 (43), 219 (72), 179 (100), 165 (35)	(Epi)gallocatechin	Su, Au, Wi, Le, Inf	Absent
3	7.0	577	MS^2 [577]: 451 (38), 425 (100), 407 (96), 305 (21), 289 (45), 287 (17)	Proanthocyanidin dimer	All samples	Absent
4	7.2	305	MS^2 [305]: 261 (12), 221 (55), 219 (77), 179 (100), 165 (26)	(Epi)gallocatechin	All samples	Absent
5	8.8	289	MS^2 [289]: 245 (100), 205 (43), 203 (28), 179 (24)	Catechin	All samples	Absent
6	9.0	353	MS^2 [353]: 191 (100), 179 (3), 173 (4), 135 (1)	Chlorogenic acid	All samples	All samples
7	9.3	865	MS^2 [865]: 739 (54), 713 (41), 695 (100), 577 (52), 451 (29), 407 (54), 405 (23), 289(19), 287 (41)	Proanthocyanidin trimer	Su, Wi, Le, Inf	Absent
8	9.5	429	MS^2 [429]: 267 (100)	Unknown	All samples	All samples
9	9.9	577	MS^2 [577]: 451 (69), 441 (17), 425 (30), 305 (100), 289 (10), 287 (8)	Proanthocyanidin dimer	All samples	Absent
10	10.1	865	MS^2 [865]: 739 (76), 695 (100), 577 (83), 451 (18), 407 (97), 287 (58)	Proanthocyanidin trimer	Su, Au, Inf	Absent
11	10.1	561	MS^2 [561]: 543(18), 435 (58), 409 (73), 425 (46), 289 (100), 271 (41) MS^3 [561→289]: 245 (100), 205 (57), 203 (30)	Proanthocyanidin dimer	All samples	Absent
12	10.9	577	MS^2 [577]: 451 (25), 441 (9), 425 (100), 407 (61), 305 (43), 289 (33), 287 (10)	Proanthocyanidin dimer	All samples	Absent
13	11.5	577	MS^2 [577]: 451 (28), 425 (10), 305 (100), 289 (4), 287 (6)	Proanthocyanidin dimer	All samples	Absent
14	12.1	289	MS^2 [289]: 245 (100), 205 (48), 203 (19), 179 (25), 161 (10)	Epicatechin	All samples	Absent
15	13.7	579	MS^2 [579]: 561 (16), 519 (16), 489 (100), 459 (99), 429 (18), 399 (50), 369 (14)	Luteolin-*C*-hexoside-*C*-pentoside	All samples	All samples
16	15.9	563	MS^2 [563]: 545 (14), 503 (15), 473 (48), 443 (100), 383 (37), 353 (43)	Apigenin-*C*-hexoside-*C*-pentoside	All samples	All samples
17	16.5	447	MS^2 [447]: 429 (14), 357 (70), 327 (100), 285 (3)	Luteolin-6-*C*-glucoside (isoorientin)	All samples	Sp, Su
18	17.0	461	MS^2 [461]: 341 (100), 313 (66), 298 (37)	Unknown	All samples	All samples
19	17.0	549	MS^2 [549]: 531 (12), 489 (26), 459 (100), 441 (13), 429 (10), 399 (64), 369 (25)	Luteolin 6-*C*-pentosyl-8-*C*-pentoside	All samples	All samples
20	17.3	563	MS^2 [563]: 503 (22), 473 (100), 443 (69), 383 (61), 353 (97)	Apigenin-*C*-hexoside-*C*-pentoside	Sp, Su, Au, Wi, Le	Sp, Su, Le, Inf
21	20.2	463	MS^2 [463]: 317 (100) MS^3 [463→317]: 271 (100), 151 (21)	Myricetin-*O*-deoxyhexoside	Sp, Au	Absent
22	20.2	431	MS^2 [431]: 341 (44), 311 (100), 283 (5) MS^3 [431→311]: 283 (100)	Apigenin-8-*C*-glu (vitexin)	Sp, Su, Au, Wi, Le	Absent
23	21.4	447	MS^2 [447]: 285 (100) MS^3 [447→285]: 285 (100), 241 (47), 151 (10)	Kaempferol-*O*-hexoside	All samples	Absent
24	22.2	417	MS^2 [417]: 399 (22), 357 (100), 327 (49) MS^3 [417→357]: 339 (100), 311 (24), 297 (82), 285 (93)	Luteolin-*C*-pentoside	Sp, Su, Au, Wi, Le	Absent
25	22.8	243	MS^2 [243]: 225 (100), 201 (50), 199 (23), 157 (20)	Unknown	Su, Inf	Absent
26	24.9	485	MS^2 [485]: 375 (100), 361 (27) MS^3 [485→375]: 357 (87), 333 (70), 329 (100), 313 (64)	Unknown	Su, Au, Inf	Absent
27	26.9	317	MS^2 [317]: 179 (100), 151 (47)	Myricetin	Sp, Su, Au, Wi, Le	Absent
28	32.1	485	MS^2 [485]: 375 (100), 357 (13) MS^3 [485→375]: 357 (100)	Unknown	Su, Au, Inf	Absent
29	36.0	285	MS^2 [285]: 285 (100), 267 (5), 243 (2), 241 (3)	Luteolin	All samples	Absent

Table 4.4 Quantification of the main compounds detected in *Cladium mariscus* (L.) Pohl (sawgrass) before PVPP sample treatment. **DE**, dry extract; **Sp,** Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences. Bold values represent the sum of each type of components. TIPC, Total Individual Phenolic Content (sum of all compounds quantified individually). --: not detected. Different superscript letters correspond to significant differences between seasons ($p < 0.05$).

No.	Assigned identification	mg g^{-1} DE					
		Sp	**Su**	**Au**	**Wi**	**Le**	**Inf**
Catechin derivatives							
3 + 4	Proanthocyanidin dimer + (epi)gallocatechin	4.5 ± 0.3^{b}	2.8 ± 0.2^{ab}	30 ± 2^{d}	9.2 ± 0.6^{c}	2.1 ± 0.1^{a}	0.84 ± 0.05^{a}
14	Epicatechin	0.57 ± 0.04^{a}	3.4 ± 0.2^{b}	5.3 ± 0.4^{c}	0.22 ± 0.02^{a}	0.17 ± 0.01^{a}	6.1 ± 0.4^{d}
Total		$\mathbf{5.1 \pm 0.3^{b}}$	$\mathbf{6.2 \pm 0.3^{b}}$	$\mathbf{35 \pm 2^{d}}$	$\mathbf{9.4 \pm 0.6^{c}}$	$\mathbf{2.3 \pm 0.1^{a}}$	$\mathbf{6.9 \pm 0.4^{b}}$
Flavonoids							
15	Luteolin-*C*-Hex-*C*-Pen	2.9 ± 0.2^{b}	2.8 ± 0.2^{b}	10.1 ± 0.6^{c}	3.4 ± 0.2^{b}	3.6 ± 0.2^{b}	0.55 ± 0.04^{a}
16	Apigenin-*C*-Hex-*C*-Pen	0.43 ± 0.02^{a}	0.43 ± 0.03^{a}	1.5 ± 0.1^{c}	0.70 ± 0.04^{b}	0.43 ± 0.03^{a}	0.32 ± 0.02^{a}
17	Luteolin-6-*C*-glucoside (isoorientin)	2.3 ± 0.1^{bc}	2.6 ± 0.1^{c}	8.3 ± 0.4^{e}	2.0 ± 0.1^{b}	3.7 ± 0.2^{d}	0.56 ± 0.03^{a}
21 + 22	Myricetin-*O*-dHex + vitexin	0.53 ± 0.04^{b}	0.41 ± 0.03^{ab}	1.7 ± 0.1^{c}	0.35 ± 0.3^{a}	0.36 ± 0.03^{a}	–
23	Kaempferol-*O*-Hex	0.87 ± 0.06^{b}	1.19 ± 0.07^{c}	3.8 ± 0.2^{d}	1.2 ± 0.1^{c}	1.36 ± 0.08^{c}	0.43 ± 0.03^{a}
24	Luteolin-*C*-Pen	0.63 ± 0.04^{a}	1.02 ± 0.06^{c}	1.8 ± 0.1^{d}	0.62 ± 0.04^{a}	0.82 ± 0.05^{b}	–
27	Myricetin	0.27 ± 0.02^{a}	0.31 ± 0.02^{ab}	0.44 ± 0.03^{c}	0.75 ± 0.05^{d}	0.39 ± 0.03^{bc}	–
29	Luteolin	0.31 ± 0.02^{a}	4.1 ± 0.2^{d}	3.5 ± 0.2^{c}	0.86 ± 0.06^{b}	0.78 ± 0.05^{ab}	6.1 ± 0.3^{e}
Total		$\mathbf{8.2 \pm 0.2^{a}}$	$\mathbf{12.9 \pm 0.3^{d}}$	$\mathbf{31.1 \pm 0.8^{e}}$	$\mathbf{9.9 \pm 0.4^{b}}$	$\mathbf{11.4 \pm 0.3^{c}}$	$\mathbf{8.0 \pm 0.3^{a}}$
TIPC		$\mathbf{13.3 \pm 0.4^{a}}$	$\mathbf{19.1 \pm 0.4^{b}}$	$\mathbf{66 \pm 2^{c}}$	$\mathbf{19.3 \pm 0.7^{b}}$	$\mathbf{13.7 \pm 0.3^{a}}$	$\mathbf{14.9 \pm 0.5^{a}}$

Table 4.5 Quantification of the main compounds detected in *Cladium mariscus* (L.) Pohl (sawgrass) after PVPP sample treatment. **DE**, dry extract; **Sp**, Spring; **Su**, Summer; **Au**, Autumn; **Wi**, Winter; **Le**, Leaves; **Inf**, Inflorescences. Bold values represent the sum of each type of components. **TIPC**, Total Individual Phenolic Content (sum of all compounds quantified individually). Different superscript letters correspond to significant differences between seasons and plant organs ($p < 0.05$).

No.	Assigned identification	mg g^{-1} DE					
		Sp	**Su**	**Au**	**Wi**	**Le**	**Inf**
Flavonoids							
15	Luteolin-*C*-Hex-*C*-Pen	0.67 ± 0.05^{cd}	0.51 ± 0.03^{b}	0.51 ± 0.04^{b}	0.61 ± 0.04^{bc}	0.77 ± 0.05^{d}	0.26 ± 0.02^{a}
16	Apigenin-*C*-Hex-*C*-Pen	0.31 ± 0.02^{ab}	0.28 ± 0.02^{ab}	0.30 ± 0.02^{ab}	0.33 ± 0.02^{b}	0.33 ± 0.02^{b}	0.26 ± 0.02^{a}
TIPC		**0.98 ± 0.05^{c}**	**0.79 ± 0.03^{b}**	**0.81 ± 0.04^{b}**	**0.94 ± 0.04^{c}**	**1.1 ± 0.05^{d}**	**0.52 ± 0.03^{a}**

4.4. DISCUSSION

Salt-tolerant plants (halophytes) have ethnomedicinal and ethnoveterinary reported uses and are considered important sources of compounds and products with multiple commercial uses. Such plants may also represent an important resource for animal management and veterinary purposes, in view of the growing need to identify alternatives to chemical ingredients in livestock production and increasing concern for animal health and better welfare practices (Oliveira et al., 2021c). In our previous work (Oliveira et al., 2021a), sawgrass aqueous/acetone extracts were rich in total phenolics and tannins, and exhibited a significant activity, especially those made from biomass collected in summer and autumn. Such results, especially the tannins levels, prompt us to evaluate the antiparasitic properties of such extracts collected along the year toward larvae and eggs from two relevant GINs, namely *H. contortus* and *T. colubriformis*. *Haemonchus contortus* resides in the abomasum, while *T. colubriformis* exist in the small intestine, and both reduce voluntary feed intake and nutrient absorption, thus reducing drastically the production of small ruminants (Hoste et al., 2016).

The extracts were more active toward larvae than eggs, most probably due to structural dissimilarities between the eggshell and the larval sheath (Mansfield et al., 1992; Hoste et al., 2015). Differences in susceptibility between eggs and adults are also attributed to the chemical components in the extracts (Araújo-Filho et al., 2018). The extracts were also more active toward *H. contortus*, which is consistent with the higher susceptibility of *H. contortus* in contrast to *T. colubriformis*, reported previously by other authors (Paolini et al., 2004; Brunet & Hoste., 2006; Quijada et al., 2015). Seasonal differences in bioactivity were observed mainly for summer and autumn samples, we questioned whether it could be (1) due to environmental effects, since the production of these metabolites is part of the plant defense machinery, to cope with the harsh settings of the dry Mediterranean climate (Di Ferdinando et al., 2014); or (2) due to differences in biochemical contents associated to the plant organs, viz. leaves and inflorescences. Thus, we proceed to investigate the *in vitro* anthelmintic properties of extracts made from these organs collected in summer (Table 4.2). The inflorescences extracts were more effective than leaves against egg hatching, for both parasite species (Table 4.2). Interestingly, despite the high activity of inflorescences sample on EHIA, this is not reflected in the seasonal samples, particularly in summer as marked differences are not

observed between samples collected in different seasons. Having in mind that an extract is a complex mixture of compounds, the latter observations can be a result of synergistic/antagonist interactions between the present metabolites or perhaps due to a dilution of the compounds of interest in the mixture (leaves and inflorescences in the summer sample) in comparison to the inflorescences extract alone.

Aiming to elucidate the role of polyphenols in the anthelmintic properties, the extracts were retested after and before treatment with PVPP, a polyphenol-binding agent. All PVPP-treated extracts retained activity toward *H. contortus* and *T. colubriformis* larvae, and egg hatching was completely restored (Figures 4.2–4.4), which agrees with previous results obtained with the summer sample (Oliveira et al., 2021b). Thus, polyphenols seem to be the main metabolites involved for the egg hatching inhibitory properties of the extracts at the highest concentration (2,500 μg mL^{-1}). In contrast, besides polyphenols, other compounds not adsorbed by PVVP seem also effective on L3 larvae exsheathment at 1,200 μg mL^{-1}.

An HPLC-ESI-MS^n comparative analysis coupled with the use of PVPP was conducted in an attempt to answer the following questions: (1) which major metabolites, removed after PVPP treatment, may be identified to exert the egg hatching inhibitory effects?; (2) which compounds may be remaining after PVPP treatment that can account for the larvae exsheathment activity?; and (3) what chemical variations occur between leaves and inflorescences extracts that may justify the significant higher anthelmintic activity of the latter samples? Our previous results obtained for the summer samples (Oliveira et al., 2021b) provided some hints to address the first two questions, yet seasonal fluctuations and organ related variations were not priorly considered. We expected that an in-depth comparative analysis of the chemical assets of seasonal and organ extracts, in combination with the biological data herein presented, enables a clarification of the bioactive metabolites of interest for AH properties and its production dynamics.

Flavonoids were the dominant compounds identified in all the extracts, especially flavan-3-ols (epigallocatechin, epicatechin **14**), proanthocyanidins (condensed tannins), luteolin, and glycosylated flavonoids (Table 4.4). Summer and autumn samples had the highest amounts of epicatechin **14** and luteolin **29**. Differences on the polyphenolic composition of plants have been demonstrated to be correlated to environmental changes, particularly in Mediterranean plants subjected to drought, high temperature and solar irradiance, high UV intensity and salt stress conditions, expected during dry seasons such

as summer and autumn (Hernández et al., 2004; Di Ferdinando et al., 2014; Gori et al., 2020). This data sustains the role of polyphenols in plant defensive and adaptative strategies to cope with the challenging Mediterranean environmental settings. For example, quercetin and luteolin derivatives act as photoprotector compounds and accumulate in response to UV radiation and increased sun irradiance (Tattini et al., 2004; Agati et al., 2011). Nevertheless, in the wild, besides environmental variations, other abiotic and biotic factors interact concomitantly, which can also affect the production of these metabolites, *e.g.,* the phenological stage of the plant. In this sense, we suspected that organ-related variations may also account for the increased **14** and **29** levels observed for these dry seasons, since the inflorescences extract (from the summer sample) also exhibit increased amounts of **14** and **29**, in contrast to leaves, supporting the former observed variations in the anthelmintic activity. Of interest is the proanthocyanidin dimer + (epi)gallocatechin 3 + 4 content in autumn, but it did not influence greatly the anthelmintic properties. In fact, high amounts of 3 + 4 are noted in all extracts except for inflorescences. Since the inflorescences extract was particularly active against GIN egg hatching, most probably these compounds are not significantly impacting the anthelmintic effects of the extracts. On the other hand, the anthelmintic value of proanthocyanidins is well-recognized in the scientific community (Mueller-Harvey et al., 2018).

Proanthocyanidins **7** and **9–13** were identified but not quantified due to low signal, limiting a complete elucidation of the contribution of tannins to the anthelmintic effects, which were present in all samples, and particularly in the summer and inflorescences extracts. Moreover, other unidentified metabolites, particularly present in inflorescences extract might also contribute to the observed effects. The *in vitro* anthelmintic effects of flavan-3-ols, proanthocyanidins and luteolin are well-described in the literature (Molan et al., 2003; Klongsiriwet et al., 2015; Quijada et al., 2015). Epicatechin and epigallocatechin suppress *T. colubriformis* larvae development by 50% at 43 μg mL^{-1}, but were less effective on egg hatching, inhibiting less than 20% up to 1 mg mL^{-1} (Molan et al., 2003). Moreover, Soldera-Silva et al. (2018) reported that epicatechin exhibits an IC_{50} value of 10 μg mL^{-1} in the larval migration test, using *H. contortus* parasites (Soldera-Silva et al., 2018). Regarding proanthocyanidins, several plant extracts rich in these metabolites have documented anthelmintic effects, influenced by the concentration, polymer size, and structural composition of these complex molecules. The most common

monomers of proanthocyanidins are catechin and epicatechin [proacyanidin (PC)-type tannins] and gallocatechin and epigallocatechin [prodelphinidin (PD)-type tannins]. Interestingly, it has been demonstrated that PD are more potent inhibitors than PC on L3 exsheathment of *H. contortus* and *T. colubriformis* (Brunet & Hoste, 2006; Quijada et al., 2015; Mueller-Harvey et al., 2018) or egg hatching of *T. colubriformis* (Molan et al., 2003), and that the addition of a galloyl group enhances activity (Molan et al., 2003). In addition to these catechin derivatives, the flavonoid luteolin **29** exhibited an IC_{50} value of 17.1 μg mL^{-1} against L3 larvae exsheathment of *H. contortus* (Klongsiriwet et al., 2015). Synergistic interactions between procyanidins and luteolin (30 mM) have been demonstrated previously, resulting in a reduced IC_{50} value of 75.9 μg mL^{-1}, in comparison to the isolated procyanidin fraction (IC_{50} = 356 μg mL^{-1}; Klongsiriwet et al., 2015). However, more recently, **29** was ineffective as a larvicidal agent at 2.5 μg mL^{-1}, against L3 larvae of *H. contortus* (Delgado-Núñez et al., 2020). These former results emphasize that flavan-3-ols, proanthocyanidins and luteolin are most likely the bioactive metabolites of *C. mariscus* extracts, as they are some of the main metabolites present. However, the contribution of flavonoid glycosides cannot be excluded, particularly because they were detected in high amounts in all extracts, except for inflorescences. Still, the considerably lower amount of flavonoid glycosides in the inflorescences extract perhaps may have unmasked the interactions with other metabolites present (e.g., proanthocyanidins and luteolin) leading to improved anthelmintic effects. Flavonoid glycosides have been identified in the chemical profile of several plant species with proven anthelmintic effects (Barrau et al., 2005; Mengistu et al., 2017; Araújo et al., 2019; Romero et al., 2020), and some authors have posed the possibility of occurring interactions of this type of compounds with GIN (Barrau et al., 2005; Alonso-Díaz et al., 2008). Barrau et al. (2005) determined that 3 flavonol glycosides (quercetin-3-*O*-rutinoside or rutin, kaempferol-3-rutinoside or nicotiflorin and isorhamnetin-3-rutinoside or narcissin) reduced the migration of *H. contortus* L3 larvae in 25–35% when applied at 1,200 μg mL^{-1}. Moreover, Sprengel Lima et al. (2021) observed that the addition of rutinose to the quercetin structure resulted in a 2-fold increase in the larvicidal activity. In addition, two flavone-C-glycosides (isoschaftoside and schaftoside) exhibited high toxicity against the parasitic nematode *Meloidogyne incognita*, leading to the death of 50% of the worms at 114.66 and 323.09 μg mL^{-1}, respectively (Du et al., 2011). Despite these limited studies, the

anthelmintic value of glycosylated forms of polyphenols remains to be elucidated as well as their interactions with aglycone molecules.

The partial inhibition of L3 larvae exsheathment in the PVPP-treated samples could be, in fact, due to the remaining content of flavonoid glycosides identified in the chemical analysis (**15–17, 19–20**). Two C-glycosyl flavonoids were quantified in higher amounts *i.e.,* luteolin-C-Hex-C-Pen 15 and apigenin-C-Hex-C-Pen 16 (Table 4.5), suggesting its potential involvement in the anthelmintic effects. The quantification of these metabolites in PVPP-treated samples did not vary greatly, except for the inflorescences extract (Table 4.5). Still, other metabolites present after PVPP treatment remain to be identified (*e.g.,* compound **8**). PVPP is widely used to adsorb polyphenolic structures from plant extracts and consequently to assess its impact on the biological activity (Barrau et al., 2005; Alonso-Díaz et al., 2008; Manolaraki et al., 2010; Vargas-Magaña et al., 2014; Mengistu et al., 2017). However, it is important to keep in mind that PVPP is generally more efficient in binding with molecules with a higher number of hydroxyl groups and that this binding is also influenced by structural substitution patterns (Doner et al., 1993). For example, Laborde et al. (2006) showed that the association of PVPP with quercetin aglycone was 4 to 5-fold stronger than that with its glucoside (quercetin-3-*O*-glucoside).

4.5. CONCLUSION

Altogether, the results of this work emphasize (1) that many factors can influence the variations in the bioactive compounds, explaining the anthelminthic properties of plants; (2) the relevance of considering abiotic factors when surveying botanical resources from the wild; (3) the potential of sawgrass as a source of bioactive metabolites against gastrointestinal nematodes, and (4) a confirmation of the anthelmintic value of a range of polyphenols in the search for alternative control options for GIN infections of ruminants. The current obtained results encouraged future work to focus on the complete elucidation of biological effects of polyphenolic glycosides, but also to explore further the interactions with other phenolic compounds and their contribution for the anthelmintic effects when mixed. In fact, we would suggest that one should be careful when assuming that the remaining activity on anthelmintic assays using PVPP treated samples is not only associated with polyphenolic compounds, and a detailed biochemical characterization by high power analyzing methods before and after the use of PVPP could help to elucidate

the bioactive metabolites of interest. In addition, the egg hatching properties of luteolin could be appraised, for a better understanding of the efficient inhibition of egg hatching by the inflorescence extracts.

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CHAPTER V

IN VIVO ANTHELMINTIC EFFECTS OF POLYPHENOL-RICH EXTRACTS FROM THE SALT-TOLERANT SPECIES *CLADIUM MARISCUS* L. POHL AND *LIMONIASTRUM MONOPETALUM* L. AGAINST GASTROINTESTINAL NEMATODES IN LAMBS

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In vivo anthelmintic effects of polyphenol-rich extracts from the salt-tolerant species *Cladium mariscus* L. Pohl and *Limoniastrum monopetalum* L. against gastrointestinal nematodes in lambs

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ABSTRACT

Background: Infections with gastrointestinal nematodes in grazing small ruminants endure as a threat to animal health, welfare and farm economy. For several decades, synthetic anthelmintics have been the cornerstone to control such parasites and limit their consequences, but novel approaches within the concept of integrated management solutions, such as bioactive plants and their associated secondary metabolites, have been increasingly explored. Recently, promising *in vitro* results were obtained with acetone aqueous extracts of polyphenol-rich salt-tolerant plants, namely *Cladium mariscus* and *Limoniastrum monopetalum*. The current study aims at obtaining the first proof of concept of the *in vivo* anthelmintic value of these two plant extracts, relying on prior *in vitro* data. **Methods:** To assess the effects of drenching the plant extracts in lambs experimentally infected with *Haemonchus contortus* and *Trichostrongylus colubriformis*, twenty lambs were divided into three experimental groups: a control group (C), a *C. mariscus* treated group (CM) and a *L. monopetalum* treated group (LM). Lambs were infected (DPI 0), drenched with extracts (DPI 30) and the experiment ended on DPI 48. Blood and fecal samples were collected regularly to assess parasitological and pathophysiological measurements. **Results:** Overall, results do not indicate significant *in vivo* parasitological effects for the two plant extracts, in the studied conditions. However, significant differences in PCV values were observed between the treated and control groups, hypothesizing potential effects on the overall animal health status, to be explored in future work. **Conclusions:** This work's outcomes are discussed based on the available *in vivo* anthelmintic works using plant extracts, and methodological challenges are appraised.

Keywords: *Haemonchus contortus*; *Trichostrongylus colubriformis*; bioactive plants; polyphenols; small ruminants; halophytes; anthelmintics

5.1. BACKGROUND

Worldwide, small ruminants' breeding systems are predominantly outdoors, relying on the use of pastures or browses, which represent an increased risk of parasitic gastrointestinal nematodes (GIN) infections due to the presence of infective third-stage larvae (L3) in the environment. GIN infections pose a significant economical and health constraint for small ruminant production and productivity (Charlier et al., 2018;2020). In particular, infections with the blood-suckling species *Haemonchus contortus* can lead to severe clinical symptoms coupled with high mortality rates (Charlier et al., 2018).

For more than 50 years, GIN control has relied mostly on the recurrent administration of synthetic anthelmintic drugs according to strategic treatment schemes; however, this method is nowadays facing strong restrictions. Firstly, due to growing concern over drug residues in animal-derived food products (*e.g.*, meat, milk) and its possible consequences to consumers' health (Charlier et al., 2024). Secondly, the long-term and intensive use of anthelmintic drugs has led to the exponential development of resistances to different commercial drug families (Rose Vineer et al., 2020; Charlier et al., 2024). The phenomenon of anthelmintic resistance is expanding worldwide, including the rise of multi-resistant isolates, as illustrated by Rose Vineer and colleagues (2020), in Europe.

To address these issues, there has been a strong impetus to explore different solutions within the novel concept of integrated management and control of GIN in ruminants, in a more sustainable manner (Torres-Acosta & Hoste, 2008; Terril et al., 2012; Charlier et al., 2024). This model proposes a so called *"basket of options"*, as a combination of several solutions focusing on three main critical stages that ensure the parasite's success: 1) the biology of worm populations; 2) the stimulation of the host responses to GIN (resistance and resilience) and 3) reduction of pasture infectivity and contact between the host and L3 infective larvae (Torres-Acosta and Hoste, 2008; Terril et al., 2012; Charlier et al., 2024).

Within this framework, bioactive plants and plant secondary metabolites have gained increasing attention: the vast plant richness and diversity, influenced by their geographic and climatic distribution, along with their phylogenetic and chemical variety, present a veritable 'pharmacy in nature. Plants represent a natural reservoir of compounds pending scientific validation and utilization, but promising to offer valuable nutraceuticals, phytotherapeutic agents, and novel scaffolds for chemical synthesis. Hoste

and colleagues (2008) have put forward a methodical framework for identifying and selecting bioactive plants and derived compounds to contribute to the GIN control in ruminants. Within this conceptual frame, the model of bioactive plants, particularly Fabaceae family members containing polyphenols, has led to a bulk of studies on their *in vitro* and *in vivo* anthelmintic properties (Shaik et al., 2006; Hoste et al., 2006; 2015; Castañeda-Ramirez et al., 2018).

Recently, polyphenol-rich salt-tolerant plants were also identified under this scheme, based on prior existing knowledge from ethnoveterinary practices and phytochemical studies (Oliveira et al., 2021a,b). *In vitro* studies confirmed the inhibitory effects of four salt-tolerant species of acetone aqueous extracts, namely *Pistacia lentiscus* L.*, Helichrysum italicum picardii* (Boiss. & Reut.) Franco 1984, *Cladium mariscus* (L.) Pohl and *Limoniastrum monopetalum* (L.) Boiss, on L3 larvae exsheathment and egg hatching processes of two GIN species, and several bioactive phenolic metabolites were identified, suggesting their involvement in the anthelmintic effects (Oliveira et al., 2021b). Within these four plants, additional investigations have been dedicated to *Cladium mariscus*, aimed at understanding the variations occurring in the anthelmintic effects and phytochemical contents, considering phenological and environmental factors (Oliveira et al., 2021c, 2022).

The translation of *in vitro* results to *in vivo* settings remains a major challenging step in the discovery pipeline of novel bioactive resources against GIN, despite *in vivo* trials remain indispensable for validating the anthelmintic efficacy of botanical products or anthelmintic drugs. Unlike monogastric animals, the gastrointestinal system of ruminants is significantly more complex, and difficulties in replicating those conditions make testing on ruminant animals the ultimate proof of concept (Torres-Fajardo et al. 2020).

Therefore, based on previous *in vitro* data, the current *in vivo* study was designed with the main aim of gathering first-time *in vivo* data on the anthelmintic properties of *Cladium mariscus* and *Limoniatrum monopetalum* acetone aqueous extracts. In line with this, efforts were directed towards the following three specific objectives: 1) to characterize the phytochemical composition of the two salt-tolerant plant extracts; 2) to analyze the effects of the oral administration of the extracts on GIN populations (egg excretion and egg development to infective larvae) and 3) its impact on lamb resilience by appraising the pathophysiological outcomes on the host.

5.2. METHODS

5.2.1. Preparation and characterization of the plant extracts

5.2.1.1. Plant collection and processing

Aerial organs of *L. monopetalum* (leaves, stems and flowers) were harvested in Ria de Alvor, Portimão (37°07'34.7"N, 8°36'02.3"W), in July 2020, whereas *C. mariscus* aerial parts (leaves and inflorescences) were manually collected in Ludo, Faro, southern Portugal (37◦ 01'03.3" N, 7◦ 59'18.1"W), in August 2020, in agreement with previous published phytochemical and anthelmintic data (Oliveira et al., 2021b,c; 2022). Plant harvesting licenses for the natural wild Portuguese territory were obtained and standard procedures of "*Instituto da Conservação da Natureza e das Florestas (ICNF)*" were followed. Samples were taken to the laboratory, washed, frozen and freeze-dried (Lyoalfa 15) for 3 days. Dry biomass was ground using a coffee and a ball miller (Retsch PM 100) until powder and stored protected from light until extraction.

5.2.1.2. Extraction of plant materials

L. monopetalum (375 g) and *C. mariscus* (525 g) dried biomass was extracted with an 80% aqueous acetone solution (1:40, *w/v*), for 16 h, at 20–25 ºC, protected from light and under magnetic stirring. Solvent extractions were made, at least, in triplicate. For each species, pooled extracts were filtered and concentrated in a rotary evaporator under reduced pressure and temperature (approx. 40 ºC). After, the acetone-free residue was freeze-dried, yielding approx. 75 g of dry extract for each species, which was later used for *in vivo* experiments.

5.2.1.3. Total phenolic (TPC) and condensed tannins (CTC) contents

The TPC of the extracts was assessed using the Folin-Ciocalteu method (Singleton et al., 1965), in 96-well plates, as previously described in (Oliveira et al., 2021c). In brief, extracts (5 µL; 10 mg mL^{-1} in DMSO) were incubated with the Folin solution (100 µL; 1:10 in distilled water) for 10 min at 20–25 °C. After the addition of sodium carbonate (100 µL; 75 g L^{-1}, in distilled water), the 96-well plate was left to incubate for 90 min, protected from light, before absorbance was measured at 725 nm using a spectrophotometer reader (Biotek Synergy 4). TPC of the samples were inferred from the standard calibration curve, using gallic acid, and expressed as gallic acid equivalents (mg GA eq. g $extract^{-1}$ dry weight).

For the estimation of CTC, the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA–HCl) method (Li et al., 1996) was performed, in 96-well plates. Plant extracts (10 µL; 10 mg mL^{-1} in DMSO), DMACA solution (200 µL; 1%, w/v in methanol) and hydrochloric acid (100 µL; 37%, v/v) were mixed and the plate incubated for 15min., before absorbance was measured at 640 nm. Again, results were inferred from a calibration curve, prepared using catechin, and expressed as catechin equivalents (mg CT eq. g $extract^{-1}$ dry weight).

For both methodologies, sample and control blanks were included and mean absorbances were subtracted to the absorbance of respective samples.

5.2.1.4. Chemical characterization through HPLC-Q-TOF-MS

Approximately 5-10 mg of each dried extract was dissolved in 1 mL of MeOH and filtered through 0.45 µm filters. The phytochemical profile of plant extracts was performed in a qualitative manner, aiming at the evaluation of the main compounds present. An Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 6530B quadrupole-time-of-flight mass spectrometer (Q-TOF MS) was used. The column was a Luna Omega Polar C18 of 150 × 3.0 mm and 5 µm particle size (Phenomenex, Torrance, CA, USA) with a Polar C18 Security Guard cartridge of 4 × 3.0 mm. The mobile phases consisted of water + formic acid 0.1% v/v (eluent A) and acetonitrile + formic acid 0.1% (eluent B). The gradient elution was: 10%–25% B in 0–25 min, 25% B in 25–30 min and 25%–100% B in 30–35 min; in the end, eluent B was returned to 10% with a 7 min stabilization time. A flow rate of 0.4 mL min^{-1} was used. The Q-TOF MS was operated in negative ion mode using an orthogonal ESI source. The parameters were: capillary voltage, 3500 V; nebulizer pressure of 45 psi; drying gas flow rate, 10 L/ min; gas temperature, 325 ◦C; skimmer voltage, 60 V; fragmentor voltage, 140 V. Continuous internal calibration was performed during analyses with the use of signals at m/z 112.9855 and 1033.9881. The MS and Auto MS/MS modes (using collision energies of 10, 20 and 40 V) were set to acquire m/z values ranging between 50 and 1200, at a scan rate of 2 and 3 spectra per second, respectively. Agilent Mass Hunter Qualitative analysis software version B.06.00 was used for post-acquisition data processing. The characterization of the compounds is detailed in Supplementary material.

5.2.2. Experimental design of the in vivo study

5.2.2.1. Production of infective larvae

Infective L3 larvae of anthelmintic susceptible isolates of *Haemonchus contortus* or *Trichostrongylus colubriformis* were cultured from feces of monospecifically infected donor sheep, recovered through the Baermann technique, and stored at 4 °C for 1-4 months, depending on species.

5.2.2.2. Animals, infection and treatment

Twenty Tarascon breed female lambs, between 3-4 months old, weighing 30-35 kg, were divided in three homogenous groups, namely: a control non-treated group (C; n=6), a group receiving the *C. mariscus* extract (CM; n=7) and a group receiving the *L. monopetalum* extract (LM; n=7). Animals were maintained indoors, in experimental facilities with concrete floors and separated boxes (12 m² each). Water, hay and mineral blocks were provided *ad libitum*.

The complete experimental design herein described is illustrated in Fig. 5.1. Lambs were bred under helminth-free conditions and tested negative for strongyle nematode infections, using the McMaster technique (Raynaud et al., 1970), seven days before infection (Fig. 5.1). Diclazuril (Vecoxan, 2.5 mg mL^{-1}, Lilly-France) was used twice at three weekly intervals, at the recommended dose of 1 mg kg^{-1} of live weight, to prevent coccidian infections and preserve animal welfare. The study lasted for 7 weeks, starting from infection (Day 0 = DPI 0). Lambs were orally infected with a single combined dose of 2000 L3 larvae of *H. contortus* and 500 L3 larvae of *T. colubriformi*s in 20 ml water. Twenty-one days post-infection (DPI 21), GIN infection was confirmed in the 3 experimental groups by fecal examination and individual measurements of eggs per gram (EPG). On DPI 30, lambs of groups CM and LM received individually respectively 9.5 grams of extract (0.3 g kg^{-1}), in 20 ml of water *per os* while lambs in the control group were drenched with 20 ml of distilled water.

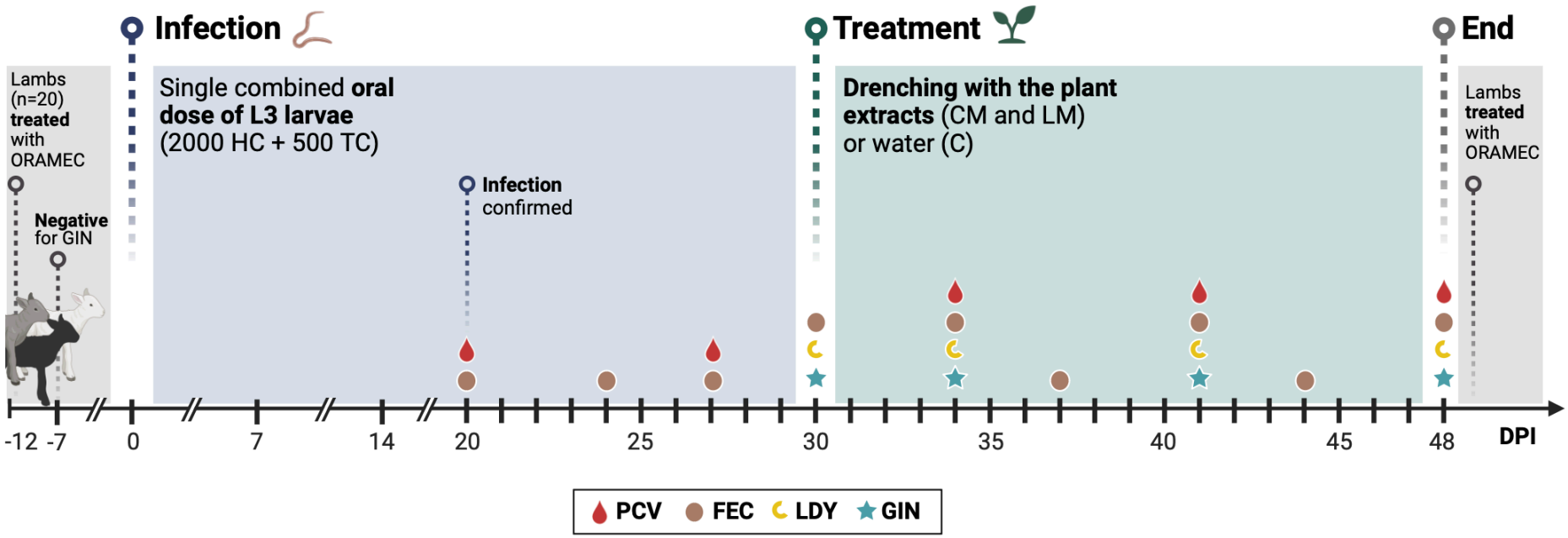

Figure 5.1 Experimental design of the study. Pathophysiological and parasitological assessments are indicated beneath the timeline, with symbols located above days post-infection (DPI), representing that sampling was performed. PCV, packed cell volume; FEC, fecal egg counts; LDY, L3 larvae development yields; GIN, relative proportions between GIN species; HC, *Haemonchus contortus*; TC, *Trichostrongylus colubriformis* larvae (Created with BioRender.com).

5.2.2.3. Infection monitoring

Individual blood samples were taken weekly (DPI 0,7, 14, 21, 27, 34, 41 and 48) by jugular venipuncture for determination of packed cell volume (PCV) levels, using a micro-hematocrit centrifuge and reader, to assess the pathophysiological effects in the three experimental groups. Parasitological evaluations were made twice a week since animal infections were confirmed (DPI 21, 24, 27, 31, 34, 37, 41, 44, 48). In brief, individual fecal samples were collected for the determination of fecal egg counts (FEC), using a modified McMaster procedure, as described by Raynaud et al., (1970), and data was expressed as eggs *per* gram of feces. Additionally, the development of eggs to L3 larvae, and the proportion of the two GIN species, were registered on DPI 30, 34, 41 and 48, aiming at evaluating the potential effects of the extracts on this GIN life process. On each date, the yield of development from eggs to L3 larvae per group was calculated as follows:

$$\frac{\text{Number of L3 collected after a 10-day larvae cultures}}{\text{Number of eggs in the fecal sample (= weight of feces X number of eggs per gram)}}$$

After larvae collection, the identification of the species, *i.e.*, *H. contortus* or *T. colubriformis*) and their relative proportions was estimated on 100 L3 larvae, on each date (MAFF, 1988).

5.2.2.4. Ethical considerations

The experiment was carried out at the National Veterinary School of Toulouse (ENVT) in the southwest of France (43°35′59'' N, 1°22′41'' E). The facilities handling and caring for the animals as well as trial protocols were approved by the French ethical and welfare rules (Comité d'Ethique en expérimentation animale´ agreement, Science et Santé Animales SSA N° 115 of Dec 15, 2014).

5.2.3. Statistical analyses

Statistical analyses were performed using the Systat 9 software. Before the statistical analyses the FEC data were normalized by using a transformation Log (n+1), as normality was not assumed for EPG. Such a transformation was not applied for PCV values which were normally distributed. FEC and PCV results have then been analyzed firstly date-by-date, using one-way ANOVA, on each DPI (0 to 48) for PCV, and on DPI 21, 24, 27, 31, 34, 37, 41, 44 and 48, for FEC. In a second step, the PCV and FEC data

obtained from DPI 21-to DPI 48 were analyzed by an analysis on repeated measurements, considering "*treatment*" and "*time*". For technical reasons, the development of eggs to L3 larvae has been applied on pooled fecal samples *per* experimental group on different dates. Therefore, the lack of repetitions restrained statistical analyses on the rate of development and on the species composition of larvae.

5.3. RESULTS

The extraction yields obtained were 21.9 ± 0.9%, for *L. monopetalum*, and 15.5 ± 1.8%, for *C. mariscus*. Both TPC and CTC values were similar between plant species. In TPC, *C. mariscus* extract exhibited 243.2 mg GA eq. g^{-1} extract DW, whilst *L. monopetalum* showed 236.8 mg GA eq. g^{-1} extract DW; in CTC, *C. mariscus* extract presented 132.3 mg CT eq. g^{-1} extract DW while *L. monopetalum* had 124.0 mg CT eq. g^{-1} extract DW.

Chemical characterization of the samples performed by HPLC-MS-Q-TOF analysis is detailed in Tables 5.1-5.2, along with the main parameters (exact mass, molecular formula, error and fragmentation), and the respective chromatographic profiles are illustrated in Fig. 5.2. Relative abundances (RA) were estimated *per* compound; however, comparisons should be cautious since ionization efficiencies differ between compounds.

C. mariscus extract was mainly comprised of luteolin C-glycosides (combined relative abundance (RA) = 25%), flavan-3-ols (epigallocatechin, epicatechin, catechin; combined RA = 15.15%), luteolin (RA = 12.33%), proanthocyanidins (combined RA = 5.76%) and apigenin C-glycosides (RA = 3.8%), accounting for 62% of the extract relative composition. Some compounds remain to be identified (**13**, **31**, **41;** combined RA = 13.81%) but malic acid (**3**), coumaric acid (**28**), taxifolin (**35**) have been herein identified for the first time in *C. mariscus* extract.

The highest contributors in the *L. monopetalum* extract (RA = 54.54%) were the methylated flavonoids sulfate **44** and **46**, followed by isorhamnetin sulfate (**43**, RA = 13.83%), ferulic acid sulfate (**17**, RA = 8.17%), pinoresinol sulfate (**42**, RA = 4.09%) and a gallic acid derivative (**22**, RA = 3.14%), in line with the previous chemical characterization performed (Oliveira et al., 2021b). Minor compounds such as isocitric (**2**), malic (**3**) and citric (**4**) acids, quercetin-*O*-hexoside (**37**) and myricetin (**45**) were not detected in *L. monopetalum* extract before (Oliveira et al., 2021b).

Table 5.1 Chemical characterization by HPLC-MS-Q-TOF of the compounds found in the extract of *Cladium mariscus*. A heat map (last column) was performed to determine the relative contribution of each compound to the whole extract (darker colour corresponds to higher abundance). For each compound, the peak area in MS mode was determined using the precursor ion $[M-H]^-$; then, the relative contribution (%) of each compound regarding the sum of the areas of all compounds was calculated. $\mathbf{t_R}$, retention time; **RA**, relative abundance (%).

No.	t_R (min)	Observed $[M-H]^-$	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
1	1.8	377.088	$C_{12}H_{22}O_{11}$	-4.97	**341.1093**, 179.0554, 161.0450, 143.0338, 119.0341, 113.0237, 101.0243, 89.0243	Disaccharide (HCl adduct)	11.55
3	2.4	133.0142	C4H6O5	0.23	**115.0032**, 71.0144	Malic acid	4.59
6	4.8	305.0671	C15H14O7	-1.57	261.0861, 219.0624, 179.0345, **125.0240**	(Epi)gallocatechin	0.85
7	7.3	577.1361	C30H26O12	-0.76	451.1040, **425.0880**, 305.0671, 289.0714	Proanthocyanidin dimer	1.27
8	7.7	305.0671	C15H14O7	-1.26	261.0661, 219.0643, 179.0345, **125.0239**	(Epi)gallocatechin	9.03
10	9.2	289.0721	C15H154O6	-1.15	**245.0829**, 205.0524, 203.0657, 179.0344	Catechin	4.29
11	9.5	353.0885	C16H18O9	-1.67	**191.0554**, 179.0332, 173.0459	Chlorogenic acid	2.15
12	9.6	865.2065	C45H38O18	-0.78	739.1536, 713.1536, 695.1377, **577.1388**, 451.0995, 425.0894	Proanthocyanidin trimer	0.15
13	10	429.1046	C18H22O12	-1.69	**267.0718**, 161.0236	Unknown	7.64
14	10.3	577.1352	C30H26O12	-0.75	451.1004, **425.0873**, 305.0654, 289.0738	Proanthocyanidin dimer	0.83
15	10.5	865.2065	C45H38O18	-1.17	739.1612, 713.1534, 695.1387, **577.1399**, 451.1191, 425.0911	Proanthocyanidin trimer	0.11
16	10.5	561.1413	C30H26O11	-0.51	447.0908, 435.1088, 409.0961, 289.0762	Proanthocyanidin dimer	1.36
18	11.3	577.1358	C30H26O12	-1.43	451.1094, **425.0873**, 305.0656, 289.0708	Proanthocyanidin dimer	0.24
19	12	577.135	C30H26O12	-0.05	451.1043, **425.0881**, 305.0656, 289.0725	Proanthocyanidin dimer	1.51
20	12.1	289.0727	C15H14O6	-2.93	**245.0808**, 205.0491, 203.0708, 179.0353	Epicatechin	0.98

No.	t_R (min)	Observed $[M-H]^-$	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
21	12.9	579.1362	C26H28O15	-0.8	561.1214, 519.1120, 489.1051, **459.0929**, 399.0703, 369.0628	Luteolin-C-glucosyl-O-arabinoside	0.32
23	14.3	579.1362	C26H28O15	-1.5	561.1212, 519.1120, 489.1036, **459.0925**, 399.0737, 369.0596	Luteolin-C-glucosyl-O-arabinoside	9.09
25	14.9	865.2065	C45H38O18	0.7	739.1603, 713.1553, 695.1410, **577.1330**, 451.1068, 425.0901	Proanthocyanidin trimer	0.29
27	16.4	563.1414	C26H28O14	-1.44	545.1211, 503.1223, 473.1103, **443.0988**, 413.0901, 383.0787, 353.0657	Apigenin-C-glucoside-C-arabinoside	2.06
28	16.6	163.0399	C9H8O3	0.93	119.0497	Coumaric acid	1.34
29	17.3	447.0941	C21H20O11	-1.76	429.0823, **357.0615**, 327.0512, 285.0401	Luteolin-6-C-glucoside (isoorientin)	10.22
30	17.3	549.1257	C25H26O14	-0.91	531.1177, 489.1017, **459.0932**, 441.0885, 399.0733, 369.0474	Luteolin-di-C-arabinoside	2.3
31	17.8	461.1095	C22H22O11	-1.29	**341.0653**, 327.0506, 313.0363, 298.0483	Unknown	3.67
32	18.2	563.1409	C26H28O14	-1.52	545.1276, 473.1073, **443.0949**, 413.0949, 383.0794, 353.0654	Apigenin-C-glucoside-C-arabinoside	0.2
34	19.9	533.1297	C25H26O13	0.48	515.1251, 473.0984, 443.0957, 413.0857, 383.0788, **353.0646**	Apigenin-di-C-pentoside	0.28
35	20.1	303.0516	C15H12O7	-2.27	**285.0410**, 125.0232	Taxifolin (dihydroquercetin)	0.17
36	20.6	431.0988	C21H20O10	-1.14	413.0889, 383.0724, 341.0669, **311.0562**, 283.0620	Apigenin-C-hexoside	1.26
39	21.9	447.0945	C21H20O11	-3.24	285.0387	Kaempferol-O-hexoside	2.87
40	22.5	417.0835	C20H18O10	-1.76	399.0740, 357.0614, **327.0523**, 297.0361, 285.0396	Luteolin-C-pentoside	3.07

No.	t_R (min)	Observed $[M-H]^-$	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
41	22.7	243.0662	C14H12O4	-0.04	201.0549, **159.0436**	Unknown	2.5
45	27	317.0308	C15H10O8	-1.83	271.0177, 178.9974, **151.0030**	Myricetin	1.46
47	36.1	285.041	C15H9O6	-1.88	---	Luteolin	12.33

Table 5.2 Chemical characterization by HPLC-MS-Q-TOF of the compounds found in the extract of *Limoniastrum monopetalum*. A heat map (last column) was performed to determine the relative contribution of each compound to the whole extract (darker colour corresponds to higher abundance). For each compound, the peak area in MS mode was determined using the precursor ion $[M-H]^-$; then, the relative contribution (%) of each compound regarding the sum of the areas of all compounds was calculated. t_R, retention time; **RA**, relative abundance (%).

No.	t_R (min)	Observed $[M-H]^-$	Molecular formula	Error (ppm)	Fragment ions	Assigned identification	RA (%)
1	1.8	377.0868	$C_{12}H_{22}O_{11}$	-2.93	**341.1085**, 179.0559, 161.0326, 143.0328, 119.0342, 113.0236, 101.0238, 89.0243	Disaccharide (HCl adduct)	1.58
2	1.9	191.0199	C6H8O7	-1.01	173.0084, **111.0083**, 87.0091	Isocitric acid	1.72
3	2.4	133.0142	C4H6O5	0.4	**115.0025**, 71.0149	Malic acid	0.32
4	2.5	191.0199	C6H8O7	-0.73	173.0088, **111.0089**, 87.0091	Citric acid	0.44
5	3.3	169.014	C7H6O5	1.27	**125.024**	Gallic acid	1.7
6	4.8	305.0669	C15H14O7	-0.59	261.0757, 219.0674, 179.0352, **125.0237**	(Epi)gallocatechin	0.97
8	7.7	305.0672	C15H14O7	-1.64	261.0800, 219.0670, 179.0354, **125.0246**	(Epi)gallocatechin	0.66
9	9.1	303.0187	C11H12O8S	-2.29	**223.0605**, 208.0370, 179.0694, 164.0480, 149.0240	Sinapic acid sulfate	1.82
17	10.6	273.0079	C10H10O7S	-1.82	**193.0501**, 178.0267, 149.0593, 134.0366	Ferulic acid sulfate	8.17
22	13.9	457.1165	C20H26O10S	1.45	329.1398, **169.0134**, 125.0237	Gallic acid derivative	3.14
24	14.3	457.1181	C20H26O10S	-1.55	**329.1397**, 245.0123, 195.0654, 165.0549	Unknown	2.05
26	15.6	411.0032	C16H12O11S	-0.55	**331.0459**, 271.0161	Unknown (sulfate adduct)	0.51
33	19.8	425.019	C17H14O11S	-0.92	**345.0615**, 330.0375, 315.0178	Dimethylated flavonoid (sulfate adduct)	0.6
37	20.7	463.0893	C21H20O12	-2.19	**301.0399**, 151.0393	Quercetin-O-hexoside	0.8
38	21.7	477.068	C21H18O13	-1.05	**301.0353**, 178.9994, 151.0026	Quercetin-O-glucuronide	1.02
42	23.7	437.0918	C20H22O9S	-1.5	357.1350, **151.0394**, 136.0162	Pinoresinol sulfate	4.09
43	24.8	395.0088	C16H12O10S	-2.46	**315.0511**, 300.0293, 271.0254	Isorhamnetin sulfate	13.83
44	25.6	425.0197	C17H14O11S	-2.93	**345.0620**, 330.0383, 315.0150, 271.0262, 287.0195	Methylated flavonoid (sulfate adduct)	25.93
45	27	317.0309	C15H10O8	-1.99	178.9978, **151.0029**	Myricetin	0.23
46	32.2	439.0357	C18H16O11S	-3.4	**359.0775**, 344.05413, 329.0299	Methylated flavonoid (sulfate adduct)	28.61
48	38.6	327.2182	C18H32O5	-1.1	291.1949, 229.1447, **211.1333**, 171.1013	Oxo-dihydroxyoctadecenoic acid	0.93
49	40.2	329.2331	C18H34O5	1.2	311.2071, 283.2588, 229.1390, **211.1339**, 171.1015	Trihydroxy-octadecenoic acid	0.9

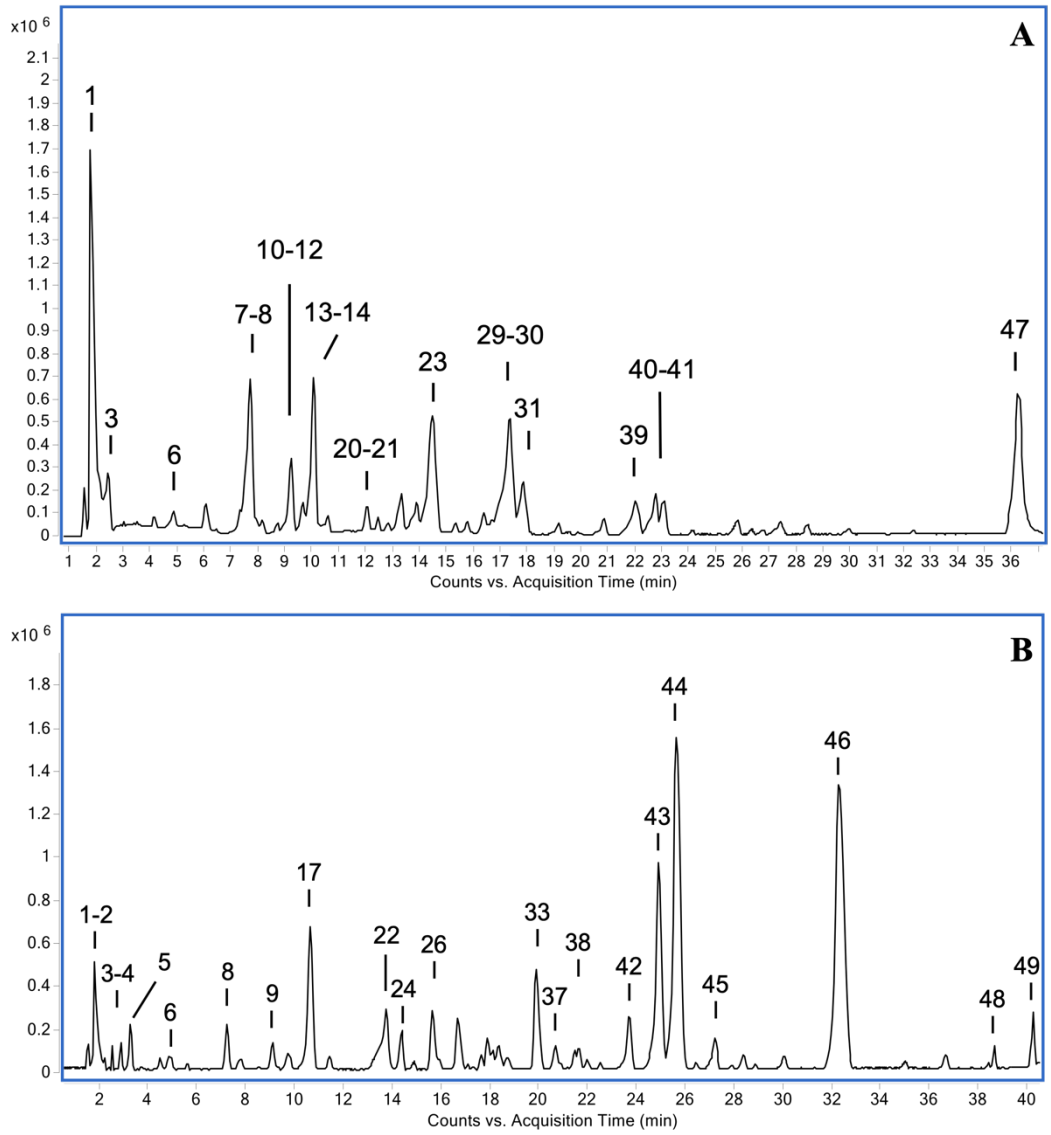

Figure 5.2 HPLC-ESI-Q-TOF-MS base peak chromatograms of *C. mariscus* (**A**) and *L. monopetalum* (**B**) 80% acetone:water extracts.

To have an overview of the potential of the extracts on infected animals, the experimental protocol was designed to assess its anthelmintic value in two frontlines, namely, 1) consequences on lamb resilience and 2) effects on the biology of parasite populations in lambs either on the worm populations in the host or on the egg development to L3 in feces coupled with GIN proportions in mixed infections. This strategy is in accordance with the conceptual frame proposed in Hoste et al. (2015).

Regarding pathophysiological measurements (Fig. 5.3), independently of the experimental group, no signs of anemia were observed, and packed cell volume (PCV) values remained within normal values (30-40 %) among groups. PCV measures the proportion of blood occupied by erythrocytes, thus, its decline is a common indicator of anemia. This measurement is particularly relevant to monitor *H. contortus* infections, due

to the species' hematophagous behavior. Overall, the comparison between groups by two-way ANOVA, based on repeated measurements, on DPI 34, 41 and 48 data, revealed statistical differences ($p < 0.05$). Results of the statistical date-by-date analyses, based on one-way ANOVA from DPI 30, indicated significant differences on DPI 34, DPI 41 ($p < 0.05$) and DPI 48 ($p < 0.01$), mainly associated with higher PCV values observed in the LM group.

A first parasitological evaluation of the consequences of administration of the two plant extracts relied on fecal egg counts (FEC; Fig. 5.3). FEC is a frequently used indirect quantitative measure to assess GIN infection levels in small ruminants and estimates future pasture contamination. In this work, no statistical differences were observed between experimental groups, either based on the repeated measurements analyses (DPI 30-48) or by referring to the date-by-date analyses. Lastly, when a co-variance analysis was applied date-by-date, using log values of EPG (n+1) on DPI 30 as a co-variable, no significant differences were observed on each date up to DPI 48.

Yields of egg development to L3 larvae and GIN species proportions were evaluated on pooled fecal cultures by groups and results are presented in Table 5.3. Although variations were observed over time in larvae yields, results were mostly homogeneous between groups, and main trends were not observed in the experimentally treated groups when compared to the control. Concerning GIN species proportions, regardless of the timepoint considered, *H. contortus* larvae were consistently dominant, ranging from 86-100 %. Having in mind the initial infection ratio (2000 L3 larvae of *H. contortus* to 500 of *T. colubriformis*) and the non-significant results obtained concerning worm biology (FEC values), these GIN proportion results are expected considering that *H. contortus* is a highly prolific egg producing parasite (daily egg output range from 5,000-15,000 eggs *per* female) while *Trichostrongylus* spp. female nematodes may expel daily only a few hundred (Coyne et al., 1991; Emery et al., 2016).

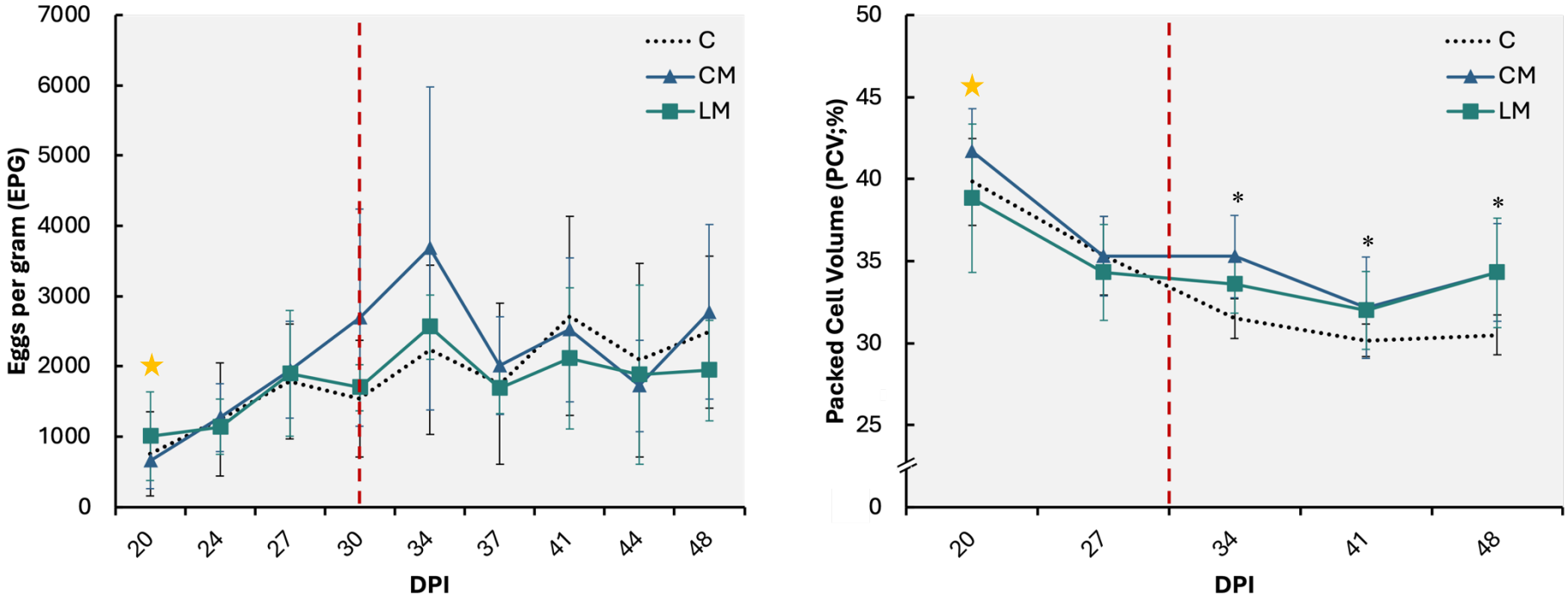

Figure 5.3 Mean EPG and PCV values and respective standard deviations in the three experimental groups in study. C, control group; CM, group receiving *C. mariscus* extract; LM, group receiving *L. monopetalum* extract. Lambs' infections were confirmed on DPI 20 (yellow star) and drenching with the plant extracts was performed on DPI 30 (red dashed vertical line). * represents statistical differences between groups, using two-way ANOVA based on repeated measurements.

Table 5.3 L3 larvae developed from eggs (%) and relative proportions (%) of *H. contortus* in relation to *T. colubriformis* L3 larvae evaluated in the three experimental groups in study: C, control group; CM, group receiving *C. mariscus* extract; LM, group receiving *L. monopetalum* extract. Lambs' infections were confirmed on DPI 20 and drenching with the plant extracts was performed on DPI 30.

Group / DPI	**30**	**34**	**41**	**48**
L3 larvae development yields (%)				
C	7.05	11.4	9.89	8.72
CM	12.2	7.94	8.2	13.4
LM	3.85	13.4	8.41	8.5
Relative proportions (%) of H. contortus *L3 larvae*				
C	95	97.7	100	99
CM	95	88	95.4	98
LM	94	89.6	98	98

5.4. DISCUSSION

Despite a panoplia of studies demonstrating the *in vitro* effects of plant extracts/fractions against different *H. contortus* and *T. colubriformis* life stages (Spiegler et al., 2017; Santos et al., 2019), there is a paucity of investigations validating the anthelmintic value in the ruminant hosts, through *in vivo* trials. In agreement, this work was designed to address this issue, by looking for the first proof of concept of the *in vivo* anthelmintic significance of hit salt-tolerant plant extracts.

Since phenolic composition can vary across harvesting periods (Oliveira et al., 2021c), the first goal was to phytochemically characterize the produced extracts to corroborate the chemical data priorly obtained with *in vitro* assessments (Oliveira et al., 2021b). In prior works, TPC levels were higher than those registered herein and *L. monopetalum* collected in July 2017 had increased tannin values (Oliveira et al., 2021b,c). Having in mind that both species were collected in the same location and season, and processed under similar methodological settings, variations in phenolic contents may result from plant species-specific adjustments in the wild, through chemical defenses, to the environmental conditions in each harvesting year (2017 vs. 2020). Nonetheless, the chromatographic profiles were comparable to those obtained earlier by HPLC-ESI-MS (Oliveira et al., 2021b;2022). Herein, the use of HPLC-Q-TOF-MS provides an additional advantage of high-resolution mass spectrometry, allowing the use of molecular formulas to confirm the identity of the compounds reported.

Although major metabolites such as flavan-3-ols, proanthocyanidins and luteolin have previously been identified as bioactive anthelmintic molecules (Molan et al., 2003; Klongsiriwet et al. 2015; Quijada et al., 2015), thereof are prime suspects as bioactive compounds, it should not be discarded that minor metabolites present may also contribute synergistically or antagonistically to the activity. As an example, myricetin derivatives have been found before in plant extracts with strong anthelmintic effects (Mengistu et al., 2017), while taxifolin was ineffective in inhibiting larvae exsheathment (Klongsiriwet et al. 2015). Indeed, Olmedo-Juaréz and colleagues (2022) inferred that when kaempferol was tested individually did not present ovicidal activity on *H. contortus* eggs, but in the presence of minor quantities of ferulic acid, the mixture was highly effective.

In the end, the phytochemical characterization of the two plant extracts was validated, being consistent with previous results obtained *in vitro* (Oliveira et al., 2021b). This supports the interest in moving forward with these extracts' *in vivo* anthelmintic

effects, as a detailed chemical characterization is fundamental whenever aiming at the development of novel standardized phytotherapeutical products against GIN.

Under the tested *in vivo* experimental conditions, mostly non-significant parasitological effects were registered. However, a significant positive effect of the *L. monopetalum* extract on lambs' haematopoiesis, noted through higher PCV values in this group. PCV is usually negatively correlated to FEC values, and both parameters are phenotypic markers of sheep resilience to GIN infection (Saddiqi et al., 2012). Although significant changes in FEC values were not observed, differences in PCV levels in relation to the control group raise the hypothesis that some bioactive plant extracts might stimulate lambs' erythropoietic system and overall animal immune status. As proven, both extracts are rich sources of a range of phenolic metabolites, well-known for their antioxidant capacity. Parasite infections trigger oxidative stress in ruminant animals (Váradyová et al., 2017; Kamel et al., 2018), which can damage erythrocytes, increase haemolysis rate and, ultimately, contribute to anaemic features (Fang et al., 2016). Fang and colleagues (2016) proved that the flavonoids orientin and luteolin can attenuate the hemolysis rate of erythrocytes under oxidative stress, preserving their cell structural integrity and, consequently, prolonging their lifespan. In addition, the inclusion of polyphenol-rich plants in the diet of infected lambs has been shown to slow the infection dynamics, improving the host's overall antioxidant status and resistance to infection (Mravčáková et al., 2019; Čobanová et al., 2020). Although it is plausible that these extracts can boost the animal's overall health status, future work is required to corroborate this premise.

The absence of significant parasitological findings in *in vivo* trials can be interpreted in two ways: either the plant extracts solely lack efficacy or adjustments to the experimental design are needed. Drawing clear conclusions is challenging, having in mind difficulties in transposing *in vitro* results to *in vivo* conditions. Indeed, plant-derived products often demonstrate better efficacy *in vitro*, most probably due to changes in bioavailability and biotransformation of active metabolites throughout the ruminant digestive system, as well as potential synergistic/antagonistic interactions between compounds (Terril et al., 1994; Gladine et al., 2007; Kim et al., 2021), reinforcing the significance of a detailed phytochemical assessment.

While established protocols exist for *in vitro* screening of plant extracts (Jackson & Hoste, 2010), recommendations for validating bioactive nutraceutical plants (Hoste et al., 2015), and guidelines for evaluating synthetic anthelmintics (Geurden et al., 2022;

Burden et al., 2024), there is still a lack of well-defined guidelines for *in vivo* trials involving plant-based extracts. Consequently, as highlighted in Santos et al. (2019), there is a limited number of *in vivo* anthelmintic studies using plant extracts whose bioactivity has been identified, and applied methodologies vary considerably, restraining comparisons and conclusions (Santos et al., 2019). Among major methodological challenges are the extracts' mode of preparation, dosage, type, and duration of treatment (*e.g.*, single *vs.* multiple doses, short-term drenches *vs*. long-term feed incorporation; Santos et al., 2019).

Herein, animals were drenched with extracts at a single low dose – thereof, one cannot assume that higher doses and multiple administration schemes would not lead to measurable anthelmintic effects. Comparably, other works also struggled to translate promising *in vitro* to *in vivo* results. For example, Oliveira and colleagues (2009) found that the ethyl acetate extract obtained from liquid of green coconut husk fiber did not present *in vivo* effects in naturally infected sheep (0.4 g kg^{-1}; three consecutive doses), although being active on *in vitro* egg and larvae assays against *H. contortus*. Eguale and colleagues (2007) tested two single doses of *Coriandrum sativum* L. 1753 aqueous extracts (0.45 and 0.9 g kg^{-1}), which were highly active *in vitro* on eggs and adult worms, against *H. contortus* experimentally infected sheep. However, discreet significant results on egg excretion and worm burden were observed, mostly in the higher dose used, while PCV values were not improved with both doses (Eguale et al., 2007). Cala et al. (2014) observed that a single dose of artemisinin (100 mg kg^{-1}), and its plant source *Artemisia annua* L. 1753 aqueous extract (2 g kg^{-1} bw) were ineffective in sheep naturally infected with GIN. The authors argued that plant-derived products should be tested in multiple doses, particularly when animals have high EPG counts, and underline that the benefits of using plant extracts could be potentially greater in combination with synthetic drugs (Cala et al., 2014).

Elsewhere, more encouraging results have also been obtained. For example, Lone et al. (2012) demonstrated an 86% and 44% reduction in egg counts with a single dose (1 g kg^{-1}) of methanolic and aqueous extracts of *Euphorbia helioscopia* L. 1753, respectively, after 18 days of post-treatment. Similarly, the crude methanol extracts of *Acacia nilotica* (L.) Willd. ex Delile bark and leaves resulted in significant reductions in fecal egg counts (FECR; 63-72%, respectively, at 8 g kg^{-1}, 12 days post-treatment; Badar et al., 2012), while a single dose (3.0 g kg^{-1}) of the methanolic extract of its fruits had a FECR of 78.5% in infected sheep (Bachaya et al., 2009). Ethanolic and aqueous extracts of the aerial

organs of *Artemisia absinthium* L. 1753, tested at 1 and 2 g kg^{-1} (single doses) were effective in reducing egg counts of naturally infected sheep, particularly the ethanolic extract at 2 g kg^{-1} (FECR of 90%, day 15 post-treatment) (Tariq et al., 2009). Recent research also reported high efficacy of hydroethanolic leaf extract of *Combretum mucronatum* Schumach. & Thonn. 1827 in goats infected with *H. contortus,* achieving up to 85.9% FECR even at lower doses (0.25 mg kg^{-1}; four consecutive days; Belga et al., 2024).

Still, according to the WAAVP guidelines (Geurden et al., 2022; Burden et al., 2024), a minimum 90% reduction in parasite counts is required for an anthelmintic to be considered highly effective. As seen above, this verge is often not reached by plant-derived products, which typically show lower efficacy compared to synthetic drugs (Githori et al., 2006; Santos et al., 2019). In addition to standardized experimental designs, revising the efficacy threshold, as suggested by Githiori and collegues (2006), may boost the validation and delivery of herbal products to be used within integrated parasite control strategies in ruminant farming systems.

5.5. CONCLUSIONS

To the best of our knowledge, this work was the first attempt to establish the *in vivo* anthelmintic value of two salt-tolerant plants' extracts, namely *C. mariscus* and *L. monopetalum*. The validation of the phytochemical profiles of the two extracts used in this *in vivo* study in comparison to previous *in vitro* works is of utmost importance for understanding the biological effects observed as well as for future standardization of potential phytotherapeutical products.

Overall, both tested extracts did not exhibit significant outcomes in the treated groups, under the studied conditions, despite a significant increase in PCV values was registered, raising the hypothesis of a potential improvement of the overall animal health status. The single low-dose treatment scheme applied had not yielded measurable anthelmintic or adverse effects, but future methodological improvements may lead to different scenarios.

In future, testing higher and/or multiple doses of these extracts, as well as understanding the bioavailability of the extracts, can significantly impact the *in vivo* anthelmintic value of these phytotherapeutical products. The lack of established protocols for the evaluation of the anthelmintic efficacy of plant extracts constrains the comparison and interpretation of currently available studies, but gathering *in vivo* evidence remains

crucial to pave the way towards the development of novel plant-based solutions to fight GIN infections.

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SUPPLEMENTARY FILES

Chemical characterization of the plant extracts through HPLC-Q-TOF-MS

The characterization of the compounds by HPLC-Q-TOF-MS was performed by accurate mass data, ion source fragmentation, MS/MS fragmentation pattern and bibliographic search. Analytical standards of catechin, chlorogenic acid, citric acid, gallic acid, proanthocyanidin dimer B1, luteolin, coumaric acid, apigenin, and quercetin were also used. Following is a brief explanation of the characterization.

Flavonoids

Catechin (compound **10**) was identified by comparison with an analytical standard. Compound **20**, with the same fragmentation pattern, was thus identified as epicatechin. Compounds **6** and **8** were the derivatives (epi)gallocatechin (Wu et al., 2012).

Compounds **7**, **12**, **14**, **15**, **16**, **18**, **19** and **25** were characterized as proanthocyanidin dimers and trimers (Kajdžanoska et al., 2010; Hamed et al., 2014). These compounds were only found in the extract of *C. mariscus*.

Compound **47** was identified as luteolin (with an analytical standard), and several luteolin *C*-glycosides were observed (compounds **21**, **23**, **29**, **30**, and **40**). The *C*-glycosides presented typical $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$ fragment ions. In a similar way (fragmentation and accurate mass data), compounds **27**, **32**, **34** and **36** were characterized as apigenin-*C*-glycosides. All the *C*-glycosides were only characterized in the extract of *C. mariscus*.

Compounds **33** and **37** were identified as quercetin-*O*-hexoside and quercetin-*O*-glucuronide, due to the neutral losses of 162 (hexoside) and 176 Da (glucuronide) and the presence of the aglycone at *m/z* 301 (fragment ions at *m/z* 179 and 151)

Compound **35** was characterized as taxifolin (dihydroquercetin) according to bibliographic information (Li et al., 2022).

Compound **39** was kaempferol-*O*-hexoside, based on the neutral loss of 162 Da (hexoside) to yield kaempferol at *m/z* 285. In kaempferol, the fragment ions at *m/z* 241 and 243, typical of luteolin, are absent.

Compound **43** was characterized as isorhamnetin ($[M-H]^-$ at *m/z* 315 and base peak at *m/z* 300), although it was sulfonated.

Compound **45** was identified as myricetin due to its molecular formula and its characteristic fragment ions at *m/z* 271 and 151.

Phenolic acids

Compounds **5**, **11** and **28** were identified as gallic acid, chlorogenic acid, and gallic acid, respectively, by comparison with analytical standards. With the typical gallic acid fragmentation (169/125), compound **22** was tentatively characterized as a gallic acid derivative.

Compounds **9** and **17** exhibited fragment ions at *m/z* 223 and 193, respectively, typical of sinapic and ferulic acid. In both cases, they were sulfonated due to the nature of *L. monopetalum* (a halophytic and metal accumulator shrub that thrives in saltmarshes under harsh biotic and abiotic stresses).

Others

Compound **1** suffered the neutral loss of 36 Da (HCl) to yield the base peak at *m/z* 341. Its fragmentation pattern was consistent with a disaccharide formed by two hexosides (probably glucose; Brudzynski & Miotto, 2011).

Compounds **2** and **4** were identified using an analytical standard of citric acid, whereas malic acid (compound **3**) was identified by the exact mass and the characteristic fragmentation 133/115 (Fernández-Fernández et al., 2010).

Compound **42** was identified as pinoresinol (sulfonated) by comparison of its experimental mass spectra with bibliographic data (Ye et al., 2005). Compounds **48** and **49** were tentatively characterized as the lignans oxo-dihydroxy-octadecenoic and trihydroxy-octadecenoic acids (Van Hoyweghen et al., 2014).

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CHAPTER VI

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

During the last decades, there has been a rising interest in harnessing the biotechnological value of salt-tolerant plants, as they hold great potential for addressing critical challenges posed by climate change and soil salinization (Bazihizina et al., 2024). Although extensive research backs up these plants' richness in added-value metabolites, which can add to the increasing demand for novel products for the food and feed, cosmetic and pharmaceutical sectors, like phenolics (Ksouri et al., 2012; Lopes et al., 2016; 2021; Attia-Ismail, 2022; El-Amier et al., 2021; Stankovic et al., 2023) and foreseen them as viable nutritional options for humans and animals (Barreira et al., 2017; Petroupolos et al., 2018; El-Amier et al., 2022; Hasnain et al., 2023), they have been largely disregarded for their utilization in the veterinary parasitology context, especially regarding GIN infections in ruminant animals. Thereof, and in retrospect to section 1.5, the main aim of this thesis was to "*explore the potential of Mediterranean salt-tolerant species as sources of added-value products for the management and control of GIN infections in ruminants*". To accomplish this goal, a funnel-like approach was employed to progressively narrow down the prospected species to the most bioactive products obtained (Figure 6.1). Hence, the present chapter is structured to trace a coherent path through the methodological steps undertaken, from species selection to *in vivo* trials, culminating in an integrated overview of the research conducted (Figure 6.1).

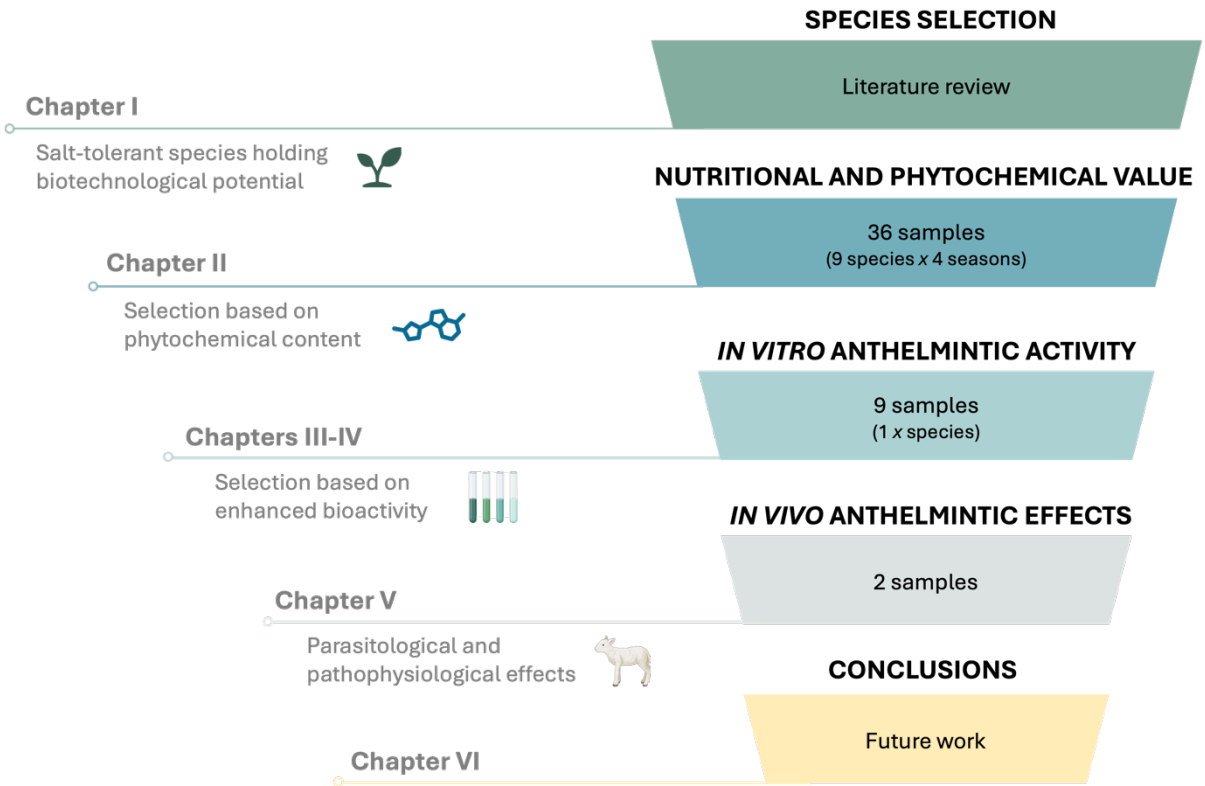

Figure 6.1 Schematic representation summarizing the step-by-step funnel-type approach undertaken throughout this dissertation (illustration icons created with BioRender.com).

Exploitation of plants and their bioactive metabolites for GIN control focuses mostly on three main key product categories: bioactive nutraceutical plants as fodders or other feed resources, aligned with the concept of anthelmintic nutraceuticals (Hoste et al., 2015; Torres-Fajardo et al., 2020); concentrated mixtures of bioactive metabolites (*i.e.,* phytotherapy or aromatherapy); and individual compounds dotted with therapeutic effects, aimed at large-scale synthesis or semi-synthesis.

As argued by Torres-Fajardo and colleagues (2020), whenever aiming at the anthelmintic use of nutraceutical plants, an interdisciplinary attitude is required. Knowledge derived from each discipline intrinsically impact others while simultaneously contributing to a more complete overview of the plant nutraceutical value (Torres-Fajardo et al., 2020). Consequently, data gathered in one subject might redirect investigations for additional applications, by creating secondary outputs, while granting the valorization of the biomass, like phytotherapeutics, nutraceutical products or as sources of bioactive compounds (Hoste et al., 2015; Torres-Fajardo et al., 2020; Figure 6.2).

The design of this dissertation was shaped by these considerations, and started by gathering traditional knowledge, either for nutrition or therapeutic ends, of several salt-tolerant species priorly identified by the Marbiotech group (CCMAR, Portugal), in the Algarve coastline (Rodrigues et al., 2014; Lopes et al., 2016).

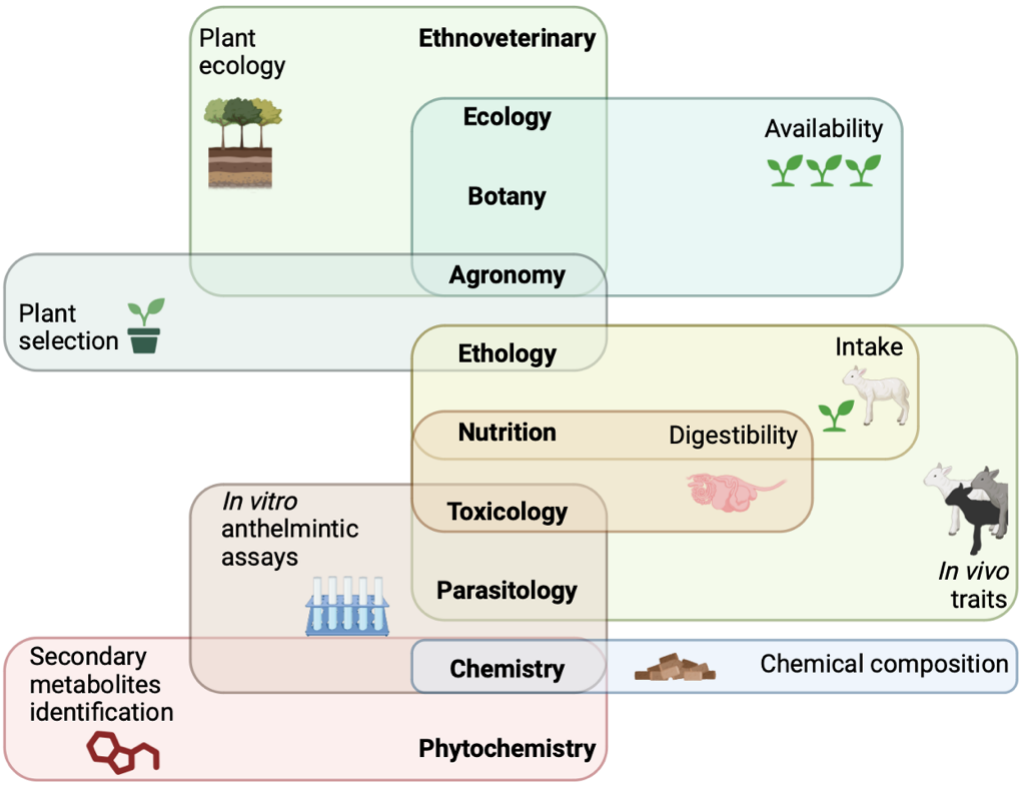

Figure 6.2 Different disciplines involved in the evaluation of anthelmintic nutraceutical plants (Adapted from Torres-Fajardo et al., 2020; Created with BioRender.com).

6.1. SALT-TOLERANT SPECIES' SELECTION

Traditional veterinary practices, honed over generations, often provide valuable insights into the use of botanical resources that can be scientifically corroborated, paving the way for the development of innovative nutritional or therapeutic solutions. However, the fragmented nature of traditional veterinary descriptions on salt-tolerant plants, along with the scarcity of reports classifying species under this botanical group, inspired the systematic review conducted in Chapter I, aiming at filling this knowledge gap (Oliveira et al., 2021a). In addition, the outcomes of the conducted review work were of outmost importance for species' selection: among 221 ethnoveterinary reports targeting ruminants, only 8 pointed out to the use of plants as feed or fodder, and nutraceutical plants were rarely mentioned; in contrast, about 92% of reports focused on therapeutic uses, including gastrointestinal disorders (section 1.4, Fig. 1.7; Oliveira et al., 2021a), sustaining the interest in phytotherapeutic applications, particularly against GIN infections.

Since the Mediterranean coastline is rich in saline environments and plant biodiversity, it was anticipated that more studies would focus specifically on highly salt-tolerant plants (*viz.*, halophytes), however, those were barely referenced (Oliveira et al.,

2021a). Still, many halophyte species occurring in the Algarve coast have relevant human ethnopharmacological uses, for example, *H. italicum* infusions have been reported as used in Portugal and Spain against inflammation, digestive disorders, intestinal parasitic infections and as an anthelmintic (reviewed by Viegas et al., 2014); infusions of *L. monopetalum* galls and leaves were used in traditional Tunisian practices as anti-dysenteric against infectious or parasitic diseases that cause painful and bloody diarrhea (Chaieb & Boukhris, 1998); and, *C. mariscus* decoctions were referred as used to treat colic in the gastrointestinal tract in Egyptian traditional medicine (AbouZid & Mohamed, 2011). This is important if one bear in mind the numerous parallels between human and animal traditional practices (McCorkle, 1986).

While an ethnoveterinary approach may serves as a valuable tool for selecting hit species, the lack of traditional animal uses, whether nutritional or therapeutical, does not necessarily imply the absence of biotechnological applications. Supporting this statement, the groundbreaking discoveries in antiparasitic nature-derived drugs awarded by the Nobel Prize in Physiology or Medicine 2015, were achieved through two distinct approaches: Tu's finding of artemisinin, an antimalarial compound extracted from *Artemisia annua*, was inspired in ancient descriptions of this herb in traditional Chinese medicinal practices (Tu, 2016); in parallel, Campbell and Ōmura discovered the anthelmintic drug ivermectin by extensively screening microbial fermentation products, derived from unique soil bacteria (Campbell, 2016).

Thus, and to ensure innovation to this thesis, the selection of plant species was widened to include not only those plants with unreported anthelmintic effects and ethnoveterinary or ethnopharmacological uses described but also combining information on their potential nutritional value, documented phytochemical content, and available biomass in the wild settings.

Upon species' selection, some scientific questions surfaced, supported by a range of studies that emphasize the influence of environmental (Bautista et al., 2016; Mahmoudi et al., 2023) and plant-related factors (Rodrigues et al., 2015) on the plant chemical composition and bioactive properties, including anthelmintic effects (Azaizeh et al., 2015). If wild plants are being surveyed, how do seasonal changes impact their nutraceutical or phytotherapeutical value and what is the optimal harvesting time? Chapter II attempted to answer these biological questions by assessing the nutraceutical and phytotherapeutical value of each plant species, at four sampling points.

6.2. NUTRACEUTICAL AND PHYTOTHERAPEUTIC VALUE

Back to the nutraceutical concept (Figure 6.2), the nutritional composition of plant resources plays a crucial role in categorizing them as *nutraceutical plants* (Hoste et al., 2015). Since protein and ash are often main limiting factors of the feeding value of salt-tolerant plants (Attia-Ismail, 2018; El Shaer, 2010; 2015; Norman et al., 2013), first screening efforts were directed towards these assets.

Overall, mainly species-specific seasonal patterns were observed. Protein contents were higher in the wet seasons, except for *M. marina* and *P. coronopus* (section 2.3.1, Fig. 2.2). The species *P. lentiscus, C. mariscus, H. italicum*, and *C. maritima* emerged as of limited nutritional interest, due to consistently low protein levels throughout seasons (section 2.3.1, Fig. 2.2). Interestingly, despite low protein content and palatability, the consumption of *H. italicum* and *P. lentiscus* by small ruminants has been reported to increase when offered in mixed feeding strategies (Rogosic et al., 2006a,b; 2008). Indeed, although its consistently high ash values herein described, *L. monopetalum* was identified in Egypt as a promising non-conventional fodder option for ruminants, but higher protein values were estimated (140 g kg^{-1} DM; El-Amier and Ejgholi, 2014). *C. soldanella, P. coronopus*, and *I. crithmoides* generally exhibited adequate protein levels for animal maintenance, though some raise concerns due to high ash or lipid contents. A key outcome was that in general species were rich sources of essential minerals such as K, Mg, Na, Fe, Mn and Zn (section 2.3.1, Fig. 2.3), which adds to the valorization of salt-tolerant species biomass throughout seasons, as feed supplements or nutraceutical products.

At this point, the use of these salt-tolerant species as nutraceutical plants seemed limited. However, either in mixed feeding schemes or as supplements, species-specific features coupled with seasonal fluctuations in nutrient composition, open the venue for multiple tailored approaches to optimize animal nutrition and health (Ben Salem & Smith, 2008). Thereof, these plants may complement conventional fodder, improve overall intake and address seasonal shortages, such as mineral deficiencies, during stressful periods like reproduction, heat stress or parasitic infections (Attia-Ismail, 2018; Masters et al., 2019). The incorporation of salt-tolerant plant ingredients in small ruminant feeds at moderate levels has shown promising results in the past, for example using *Atriplex barclayana, Suaeda esteroa* Ferren & S.A.Whitmore, 1983 and *Salicornia bigelovii* Torr., 1859, in Mexico (Swingle et al., 1994; 1996) or *Atriplex nummularia* Lindl., 1848,

Beta vulgaris L. *Pennisetum glaucum* (Maire) Brunken, in Egypt (Abo Bakr et al., 2020). Still, careful considerations of ash levels, along with the potential impact of secondary metabolites and further detailed assessments on fiber and energy are essential to be investigated, aiming at a complete picture of their nutritional value and avoidance of deleterious effects on intake and digestibility. Nonetheless, in the light of climatic change impacts, water scarcity and increased saline degraded lands in the Mediterranean region, there is still opportunity for the strategic integration of salt-tolerant species to support sustainable animal management practices.

Regarding their phytochemical value, as detailed in section 1.3.4, phenolics are in the epicenter of the anthelmintic discovery pipeline against GIN in ruminants. Previous studies have generally considered extracts with TPC values exceeding 20 mg GAE g^{-1} extract as good sources of these compounds (Kähkönen et al. 1999; Rodrigues et al. 2015), and herein, except for *M. marina* and *I. crithmoides*, all studied species met this criterion (section 2.3.2, Fig. 2.4). Seasonal dynamics in phenolic contents varied between species and were correlated to their antioxidant effects: *H. italicum* had higher TPC in autumn, *L. monopetalum* and *C. soldanella* in spring, *P. lentiscus* in autumn/winter, *C. mariscus* in summer/autumn, *P. coronopus* in winter and *C. maritima* only decreased in summer (section 2.3.2, Fig. 2.4; Table 2.1). Most species accumulated higher amounts of flavonoids in spring/summer, except for *I. crithmoides* and *P. lentiscus*, which increased in autumn. Condensed tannins were detected in *L. monopetalum*, *C. mariscus*, and *P. lentiscus* samples (section 2.3.2, Fig. 2.5): in a nutraceutical perspective, as anti-nutritional compounds, it is assumed that high tannin concentrations limit feed intake and nutrient digestibility, as it is the case of *P. lentiscus* (approx. 20% DM; Decandia et al., 2000). Thereof, as nutraceutical plants, *C. mariscus* tannin contents would be safely within range (3-4%; data not shown) but *L. monopetalum* intake may be dependent on seasonality (2-7%; data not shown).

The phytochemical profiling of the selected most bioactive samples (section 2.3.3, Fig. 2.6, Table 2.2) revealed their chemical complexity and species-related features. Several bioactive molecules were identified in all samples, including 14 compounds for which the *in vitro* anthelmintic activity has been previously assessed (Brunet & Hoste 2006; Klongsiriwet et al. 2015; Mancilla-Montelongo et al. 2019; Sprengel Lima et al. 2021). In LEIA, against *H. contortus* larvae, ferulic acid exhibited an EC_{50} value of 7.8 μg mL^{-1}, quercetin of 21 μg mL^{-1}, *trans*-ferulic acid of 20.6 μg mL^{-1}, *trans*-cinnamic acid of 34.4 μg mL^{-1} and chlorogenic acid of 92.4 μg mL^{-1} (Klongsiriwet et al., 2015;

Mancilla-Montelongo et al., 2019). In EHIA, outstanding results have been obtained with the phenolic acids ferulic, caffeic and gallic (0.56-4.93 μg mL^{-1}), whilst flavonoids showed much higher EC_{50} values (663-1260 μg mL^{-1}) against *H. contortus* egg hatching (Sprengel-Lima et al., 2021). These 3 phenolic acids were also the most efficient in neutralizing *H. contortus* larval development (22-33 μg mL^{-1}), followed by chrysin (58 μg mL^{-1}), rutin (104 μg mL^{-1}), quercetin (231 μg mL^{-1}), morin (448 μg mL^{-1}) and ourateacatechin (989 μg mL^{-1}; Sprengel-Lima et al., 2021). This phenolic richness of the screened extracts envisions phytotherapeutical applications of these species for their anthelmintic properties but may also display additional impacts on animal health and productivity (Olagaray & Bradford, 2019).

As discussed in section 1.3.2, in wild settings, salt-tolerant plants are subjected to many abiotic and biotic factors, which influence the biosynthesis of phenolic compounds. As a side note, herein, focus was on *when* seasonal variations occur and *how* they impact biotechnological value rather than delving into *why*. Although ecological traits were left outside of this thesis scope, unravelling these configurations behind the plants' phytochemical profile is worthy of future investigations, as portrayed in Figure 6.2, particularly whether aiming to optimize cultivation processes, to unveil in-depth relationships between bioactive metabolites of interest and optimize their production.

In the end, findings of Chapter II add to the state of the art by providing an overview on each species' chemical and biological attributes, its seasonal dynamics and subsequent impact on their nutraceutical or phytotherapeutical significance. Their phytochemical content was validated, and the season that yielded greater amounts of these bioactive molecules identified for each species under study. As *in vitro* anthelmintic studies, reliant on microscopy techniques, are laborious and time-consuming, one sample *per* species followed to *in vitro* assays, based on those displaying the combined highest phenolic content and antioxidant properties reported during this chapter, foreseeing enhanced bioactivity in Chapter III.

6.3. *IN VITRO* ANTHELMINTIC ASSESSMENTS

In vitro methodologies are first-line choices to determine the anthelmintic effects of plant resources, with several assays being available targeting different aspects of GIN life cycle (see section 1.3.1., Fig. 1.4.). However, it cannot be assumed that an active extract on one *in vitro* assay will be similarly effective on another, as variations on the susceptibility among parasite life stages and life processes occur (Mansfield et al., 1992;

Alonzo-Díaz et al., 2011; Sprengel Lima et al. 2021; Oliveira et al., 2021c; 2022), even when testing commercial anthelmintic drugs (Munguía et al., 2022). In line with this, the *in vitro* assays chosen for this work focused on transitory processes occurring between the parasitic and free-living nematode stages, namely L3 exsheathment and egg hatching (section 1.3.1., Fig. 1.4.).

Chapter III was crucial to provide groundbreaking results to this thesis, by disclosing the anthelmintic activity of salt-tolerant plants. Aside from *P. lentiscus*, which has been priorly identified as active on larval migration and exsheathment processes (Manolaraki et al., 2010; Azaizeh et al., 2013), to the best of our knowledge, none of studied species were previously investigated for their anthelmintic potential. Amongst all, *P. lentiscus*, *L. monopetalum*, *C. mariscus* and *H. italicum picardii* extracts stood out as the most bioactive (section 3.3.2, Table 3.3; Oliveira et al., 2021c), in agreement with the phytochemical results obtained in Chapter II. The HPLC-ESI-MS^n profile of the extracts revealed that mostly flavonoid glycosides and galloylquinic acid isomers occur in *P. lentiscus*; caffeoylquinic and dicaffeoylquinic acids and quercetin glycosides in *H. italicum picardii*; proanthocyanins, phenolic acids, and luteolin in *C. mariscus*; and sulphated and/or methylated flavonoids in *L. monopetalum* (section 3.3.4, Fig. 3.4, Tables 3.4-3.7; Oliveira et al., 2021c). The identification of several individual or group of metabolites with previously reported anthelmintic effects backed up the results obtained, as discussed in Chapter III, section 3.3.4. (Molan et al., 2003; Klongsiriwet et al., 2015; Quijada et al., 2015; Soldera-Silva et al., 2018). However, the combined PVPP/HPLC-ESI-MS^n approach provided additional important remarks, being fundamental to an integrated interpretation and inference of the potential bioactive metabolites involved in the anthelmintic effects. Recently, this approach has been replicated in similar works (Meza Ocampos et al., 2023).

Main findings were that, while in EHIA, all PVPP-treated samples restored egg hatching to control levels, pointing towards polyphenols as main responsible, whilst L3 exsheathment was not always completely re-established (section 3.3.3, Figs. 3.2-3.3; Oliveira et al., 2021c). This has been observed in other works exploring polyphenol-rich extracts, using PVPP as a polyphenol-binding polymer (Barrau et al., 2005; Manolaraki et al., 2010; Meza Ocampos et al., 2023). At this point, the core questions were which metabolites are present in non-treated samples that inhibit egg hatching? and which compounds remaining in the extracts after PVPP treatment are being active on LEIA?

Polyphenol inhibitors, such as PVPP, have been broadly used as a tool for *in vitro* studies to investigate the role of polyphenols in the anthelmintic effects (Barrau et al., 2005; Manolaraki et al., 2010; Vargas-Magaña et al., 2014; Meza Ocampos, 2023). However, polyphenol-PVPP binding depends on several factors, among them the number of hydroxyl groups and aromatic rings, methyl- and glycosyl- substitution patterns and coplanarity of the flavonoid ring C (Donner et al., 1993; Verza et al., 2008; Durán-Lara et al., 2015). As shown by Durán-Lara and colleagues (2015), this inhibitor has higher affinity to quercetin, catechin and epicatechin in relation to simple phenols. Laborde et al. (2006) found that PVPP-quercetin aglycone binding was 4 to 5-fold stronger than that with its glucoside (quercetin-3-*O*-glucoside). Yet, independently of the amount of PVPP in use, its binding to polyphenols lacks specificity, which was also confirmed in more complex matrices such as plant extracts (Verza et al., 2008).

These works back up the overall outcomes of this thesis, since treated samples were mainly composed of remaining polyphenolic molecules with substitution patterns and simpler phenolic structures, such as chlorogenic and caffeic acid (section 3.3.4, Fig. 3.4, Tables 3.4-3.7; Oliveira et al., 2021c). The possibility of flavonoid glycosides to be active on GIN has been raised before, but only few disclosed the activity of such molecules. The activity of three flavonol glycosides (quercetin-3-O-rutinoside, kaempferol-3-rutinoside, and isorhamnetin-3-rutinoside) on *H. contortus* larval migration was confirmed (1200 µg mL; Barrau et al., 2005). Barrau and colleagues (2005) underlined the structural resemblances between flavonol glycosides and condensed tannins, suggesting a closely related mode of action. In another study, two flavonoid glycosides, luteolin-7-*O*-ß-glucopyranoside and quercetin-3-*O*-ß-glucopyranoside, were identified as responsible for the anthelmintic effects of *Vicia pannonica* Crantz, 1769, var. *purpurascens*, showing 100% effectiveness in inhibiting the motility of *Trichostrongylus* parasites in rumen fluid at a concentration of 1 mg mL^{-1} (Kozan et al., 2013). More recently, Sprengel-Lima et al. (2021) observed that the glycoside rutin had a 2-fold increase in activity on larvae development in comparison to the aglycone quercetin. Either way, more research needs to be conducted for a complete overview of the structure-activity relationships of phenolic glycosides and respective aglycones. This is of particular interest for *in vivo* settings, considering the findings of Berger et al. (2012), who determined that the bioavailability of quercetin, after intraruminal application in cows, was clearly greater if derived from rutin other than the aglycone.

As a side note, and distancing from the findings of Chapters II and III, the species identified as having more potential as nutraceutical plants—such as *M. marina* and *C. soldanella*, due to their nutritional attributes—demonstrated no significant anthelmintic effectiveness in *in vitro* assays. Conversely, the most bioactive species, including *P. lentiscus*, *L. monopetalum*, *H. italicum*, and *C. mariscus*, displayed limited nutritional value in Chapter II. In retrospect to the opening of this general discussion (Figure 6.2), the anthelmintic value of these plants headed towards their phytotherapeutical exploitation instead of nutraceutical.

Supported by the data priorly obtained, Chapter IV examined the anthelmintic usefulness of *C. mariscus* and its seasonal and plant-related variations. The aerial organs of sawgrass primarily consisted of leaves and inflorescences, the latter being collected in summer and, to a lesser extent, in autumn. It was theorized that inflorescences might have contributed to higher phenolic content, antioxidant, and anthelmintic activities in the summer sample (Chapters II-III), raising the question of whether anthelmintic activity was following seasonal fluctuations of phenolic contents or if this was linked to its plant organs. It is reported that distinct plant organs display different phenolic profiles as well as antioxidant properties (Falleh et al., 2008; Rodrigues et al., 2015). Also, seasonal variations in *P. latifolia*, *P. lentiscus*, and *I. viscosa* extracts revealed that anthelmintic activity did not entirely align with changes in phenolic contents, suggesting other crucial factors, such as species-specific phenolic composition, the presence of other metabolites, and plant-related factors (Azaizeh et al., 2015). Still, to the best of knowledge, works concerning changes on anthelmintic effects amongst plant organs and its relation to season, on the same plant species, were not conducted before.

Whenever seasonal differences were significant, the summer sample displayed greater anthelmintic efficacy. In agreement, the inflorescences extract was significantly more effective than the leaves extract on EHIA, against both GIN species, and on LEIA against *H. contortus* (section 4.3.1, Tables 4.1-4.2; Oliveira et al., 2022). Summer, autumn and inflorescences samples had the highest amounts of epicatechin and luteolin, in contrast to leaves, whilst flavonoid glycosides were abundant in all samples, but its occurrence was more pronounced in leaves than in inflorescences (section 4.3.3; Table 4.3-4.5; Oliveira et al., 2022). These compounds are usually produced in leaves, shielding plants against solar irradiation and UV radiation (Groenbaek et al., 2019), thus season and light influence their composition and concentration (Schmidt et al., 2010). In PVPP-treated samples, the inflorescences extract exhibited the lowest levels of flavonoid

glycosides (section 4.3.3, Table 4.5; Oliveira et al., 2022), but remained active on LEIA (section 4.3.2, Figs 4.2-4.3; Oliveira et al., 2022), reinforcing these metabolites potential anthelmintic effects. On the other hand, could flavonoid glycosides have antagonized the egg hatching inhibitory effects of catechin derivatives and luteolin? This is conceivable, and answers remain to be pursued, as the inflorescence extract had lower amounts of these compounds and still was the most bioactive in EHIA, while summer and leaves extracts had increased EC_{50} values.

In sum, Chapter IV complemented results obtained in Chapter III, by gathering knowledge on *C. mariscus* bioactive properties, validating its anthelmintic effects throughout the year, and establishing the phytotherapeutic significance of different plant organs.

6.4. *IN VIVO* ANTHELMINTIC EFFECTS

Upon Chapter III and IV, two species were highlighted as the most promising in *in vitro* evaluations after *P. lentiscus*, *viz.*, *C. mariscus* and *L. monopetalum.* Following the thread (Figure 6.1), those were selected to gather, first *in vivo* evidence of their anthelmintic effects. Despite the phytochemical profile of both extracts being in accordance with those previously determined in Chapters III and IV, parasitological outcomes were mainly non-significant for both treated groups. Only exception was PCV values, which displayed differences between groups from day post-infection (DPI) 34 onwards, despite still within normal ranges (section 5.3, Fig. 5.3), paving the way for future investigations on these extracts' potential improvement of the overall animal immune and antioxidant status. Additionally, the applied orally drenched extract dose did not show any apparent animal toxicity.

The lack of significant parasitological results could be explained by difficulties in transposing *in vitro* results to *in vivo* experiments. Though *in vitro* methods are a valuable tool for screening purposes, *in vivo* trials are indispensable for validating the anthelmintic efficacy of the botanical products under study. However, since the complex chemical and biological dynamics of the ruminant gastrointestinal environment can only be fully replicated in *in vivo* trials (Villalba & Provenza, 2010), results from *in vitro* assays do not necessarily foreseen activity under *in vivo* conditions. This is exemplified by previous studies using plant extracts that also achieved non-significant to moderate outcomes (Eguale et al., 2007a,b; Oliveira et al., 2019; Cala et al., 2014; Table 6.1). In fact, in the ruminant gastrointestinal tract, the bioactive metabolites in question may interact with

nutrients or other secondary metabolites, influencing their bioactivity and bioavailability, as for example, tannin-proteins complexes (Villalba & Provenza, 2010). Rumen microbial communities have also a major role in the digestive process, influencing the biotransformation and degradation of phenolic metabolites (Kim et al., 2021), potentially altering biological effects.

Another theory explaining the absence of relevant *in vivo* findings in this work could be related to experimental design features. As discussed by the end of Chapter V, while recommendations for the *in vivo* testing of nutraceutical plants (Hoste et al., 2015) and guidelines for anthelmintic drugs are available (Geurden et al., 2022; Burden et al., 2024), it is missing a distinct protocol for phytotherapeutical products. Thereof, as illustrated in Table 6.1., the applied methodological schemes, such as animal infection protocols, dose and frequency of administration, vary widely in the available literature, limiting interpretations. As an example, some trials started with lower initial egg counts (range between 100-750 EPG; Mohammadian et al., 2024; Ademola et al., 2004) while EPG counts at day 0 were much higher for others (> 5000 EPG; Toklo et al., 2023; Morais-Costa et al., 2016; Cala et al., 2014); the frequency of administration ranges from single dose up to four doses, and the doses applied are between 7.5-8000 mg kg^{-1} (Table 6.1). In this sense, it is plausible that other combinations of dose and frequency of administration of the *C. mariscus* and *L. monopetalum* extracts in study could lead to significant outcomes.

In the end, although promising *in vitro* results were obtained for the studied plant extracts, the *in vivo* effects of these products remain to be fully clarified and validated. In future, different methodological conditions should be tested and updated information related to the bioavailability of the potential bioactive compounds identified in the extracts should be considered. As argued by others, this lack of standardized protocols and phytotherapeutic products continues to pose an obstacle to the development and launching of novel veterinary phytotherapeutics in Europe (Blanco-Penedo et al., 2018; Tamminen et al., 2018).

Table 6.1 *In vivo* trials conducted to assess the anthelmintic effects of plant-derived extracts in small ruminants infected with gastrointestinal nematodes, and respective methodological parameters used. **Plant organs:** F, fruits, S, seeds; L, leaves; Ba, bark; Bu, Bulb; W, whole. **Solvents:** W, water; ET, ethanol, M, methanol; EA, ethyl acetate; WM, aqueous methanol; WET, hydroethanolic. **Animals:** S, sheep; G, goats. **Infection:** N, natural infection; E, experimental infection. **n.d.**, not detailed. **FECR**, Fecal Egg Count Reduction. **DPT**, days post-treatment.

Species	Organ	Animals	Solvent	Infection	Dose	Mode	Frequency	Observations	Ref.
Lachesiodendron viridiflorum (Kunth) (syn. *Piptadenia viridiflora)*	L	S	W	E	283 mg kg^{-1}	Gavage	Three	DPT 21: FECR 32.90%	[1]
Hedera helix L.	F	S	W	E	1.13 and 2.25 g kg^{-1}	Drench	Single	DPT 2: FECR 46.17% with the highest dose	[2]
Coriandrum sativum L.	S	S	W	E	0.45 and 0.9 g kg^{-1}	Drench	Single	DPT 2: FECR 24.5% with the highest dose	[3]
Azadirachta indica A.Juss.	S	S	M	N	1 and 3 g kg^{-1}	ND	Single	DPT 14: FECR 40.2% with the highest dose	[4]
Nicotiana tabacum L.	L	S	W / M	N	1 and 3 g kg^{-1}	ND	Single	DPT 5: FECR 73.6% (methanol) and 49.4% (water) with the highest doses	[5]
Spondias mombin L.	L	S	ET	N	0.125, 0.25 and 0.5 g kg^{-1}	Drench	Single	DPT 12: FECR 5.6-15%, dose dependent	[6]

Species	Organ	Animals	Solvent	Infection	Dose	Mode	Frequency	Observations	Ref.
Khaya senegalensis (Desr.) A.Juss.	Ba	S	ET	N	0.125, 0.25 and 0.5 g kg^{-1}	Drench	Single	DPT 12: FECR of 35.3-71.8%, dose dependent	[7]
Artemisia absinthium L.	W	S	ET / W	N	1 and 2 g kg^{-1}	Oral	Single	DPT 12: FECR 73.44% and 80.49% (water); FECR 82.85-90.46% (ethanol)	[8]
Allium sativum L.	Bu	S	W	N	5g animal	Oral	Single	DPT 21: FECR 56.9%	[9]
Combretum glutinosum Perr. ex DC.	Ba	S	W	E	0.1, 0.2 and 0.4 g kg^{-1}	Oral	Single	DPT 21: FECR 78.55% (0.1 g kg^{-1}), 31.22 % (0.2 g kg-1), 62.75 % (0.4 g kg^{-1})	[10]
Ziziphus nummularia Wight & Arn. / *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb., 2008 (syn. *Acacia nilotica*)	Ba / L	S	M	N	1, 2 and 3 g kg^{-1}	Oral	n.d.	DPT 13: FECR of 84.7% (*Z. nummularia*; methanol) and 78.2% at 3.0 g kg^{-1} (*A. nilotica*; methanol)	[11]
Trianthema portulacastrum L. / *Musa paradisíaca* L.	W	S	WM	N	1, 4 and 8 g kg^{-1}	Oral	Single	DPT 15: 85.6% (*T. portulacastrum*) and 80.7% (*M. paradisiaca*)	[12]
Cocos nucifera L.	F	S	EA	N	0.4 g kg^{-1}	Oral	Three	Efficacy on worm burden of 9%	[13]
Artemisia annua L.	L	S	W	N	2 g kg^{-1}	Oral	Single	DPT 15: 19.3%	[14]

Species	Organ	Animals	Solvent	Infection	Dose	Mode	Frequency	Observations	Ref.
Euphorbia helioscopia L.	W	S	W / M	N	1 g kg^{-1}	Oral	Single	DPT 18: FECR 44.15% (water), 86.07% (methanol)	[15]
Vachellia nilotica (L.) P.J.H.Hurter & Mabb., 2008 (syn. *Acacia nilotica*)	Ba / L	S	WM	N	4 and 8 g kg^{-1}	Oral	Single	DPT 12: FECR 72.01% (bark) and 63.44% (leaves) at highest dose	[16]
Combretum mucronatum Schumach. & Thonn.	L	G	WET	E	0.25, 0.5, 1 g kg^{-1}	Drench	Four	DPT 21: 85.32-98.76%, dose dependent	[17]
Pelargonium quercetorum Agnew	E	G	L	N	7.5 mg kg^{-1}	Herbal bolus	Single	DPT 14: FECR 63.41%	[18]

[1] Morais-Costa et al., 2016; [2] Eguale et al., 2007a; [3] Eguale et al., 2007b; [4] Iqbal et al., 2010; [5] Iqbal et al., 2006; [6] Ademola et al., 2005; [7] Ademola et al., 2004; [8] Tariq et al., 2009; [9] Kanojiya et al., 2015; [10] Toklo et al., 2023; [11] Bachaya et al., 2009; [12] Hussain et al., 2011; [13] Oliveira et al., 2009; [14] Cala et al., 2014; [15] Lone et al., 2012; [16] Badar et al., 2011; [17] Belga et al., 2024; [18] Mohammadian et al., 2024.

6.5. CONCLUSIONS AND FUTURE PERSPECTIVES

Any doctoral dissertation is expected to provide answers to scientific questions, yet it often generates numerous more along the process. In that sense, this sub-section aims at answering the biological queries initially raised by the end of Chapter I, while identifying limitations and future work to be pursued.

Can these plants be considered as nutraceutical plants?

In part, yes. The sole use of individual species as nutraceutical plants seems limited, but depending on season and species, several samples displayed adequate protein, ash and mineral contents. However, as depicted in Figure 6.2, unraveling the nutraceutical value of a botanical resource proves to be a multifaceted endeavor. Further studies on ruminant nutrition, ethology, and agronomy, are required to complement the present data to fully ascertain the nutraceutical potential of these species. As an example, despite of the phytochemical content, anthelmintic evaluation, and macro- and micronutrient contents, Hoste and colleagues (2015) underline other decisive features in the nutraceutical concept such as fiber fractions, voluntary feed intake, *in vitro* and *in vivo* digestibility, and overall impact on animal production. Fiber and *in vitro* organic matter digestibility were quantified for *C. mariscus*, confirming their limited nutritional usefulness. Still, out of curiosity, generally wild salt-tolerant plants had higher fiber contents than cultivated siblings to face with environmental constraints (Barreira et al., 2017; Castañeda-Loaiza et al., 2020; Lima et al., 2020). Since Chapter II intended screening purposes and the phytotherapeutic approach was pursued, some of these parameters were not generally assessed, but are essential to be evaluated later, if aiming the utilization of these plants for such end. Nonetheless, the nutritional-related outputs generated in this work shed light on the versatility of these species, extended beyond the context of GIN infections, as it is case of their noteworthy mineral content and antioxidant features, opening the opportunity for their exploitation as feed supplements, nutraceutical products or to be used in mixture with other conventional fodders.

Are these plants rich sources of bioactive secondary metabolites of nutraceutical or phytotherapeutic interest?

Yes. Phytochemical investigations conducted and validated along this thesis chapters (II-IV) unveil that most species were sources of metabolites of phenolic nature, with antioxidant and anthelmintic features, particularly *P. lentiscus*, *C. mariscus*, *L. monopetalum* and *H. italicum picardii*. However, species-specific seasonal patterns on the phenolic profile and content were observed and should be considered for future exploitation of these species.

Are the plant-derived products able to disrupt the biological cycle of gastrointestinal nematodes?

Yes. Chapters III and IV were fundamental to deliver breakthrough results for this dissertation by confirming that salt-tolerant plant extracts can disrupt the biological cycle of GIN *in vitro*. *P. lentiscus*, *C. mariscus*, *L. monopetalum* and *H. italicum picardii* stood out as the most bioactive plant extracts, either against L3 larvae exsheathment or egg hatching of two clinically relevant GIN species. In addition, *C. mariscus* extracts showed promising *in vitro* results throughout seasons and among organs, widening its potential exploitation and application.

a) Are phenolic compounds involved in the anthelmintic effects?

Yes. Chapters III and IV provided valuable insights about the potential bioactive metabolites of the active extracts as well as their contribution for the therapeutic properties. The PVPP combined approach brought novelty to the work, acting as an unspecific fractionation of the samples, allowing *before* and *after* treatment comparisons and backing up the anthelmintic value of different phenolic structures.

However, except perhaps for the tannin group, plenty remains to be investigated and understood for an effective use of these metabolites in GIN infections. The synergistic and antagonistic interactions between phenolic compounds in botanical mixtures along with their individual anthelmintic value, structure-activity and bioavailability/biotransformation studies, are topics awaiting to be completely uncovered. Also, the chemical scaffolds of some major compounds detected in the active extracts were not disclosed, opening a research opportunity for the isolation and identification of these molecules and investigations around their anthelmintic features.

Lastly, rumen microbiome plays a significant role in ruminant digestion, and recent works point out to significant changes occurring on the ruminal microbiota of non-

infected, GIN-infected and GIN-infected animals supplemented with other plant resources (Corrêa et al., 2020; Fan et al., 2024). Understanding the intricate microbiome interactions when GIN and anthelmintic products/metabolites are in the equation, hold tremendous potential. In future investigations on how these microbial communities change during GIN infections and how to modulate them to favor desired outcomes, can boost the utilization and effectiveness of the current anthelmintic products as well as the development of novel preventive and therapeutic solutions.

b) *Does seasonality impact the nutraceutical or phytotherapeutic value of these species?*

Yes. Seasonal fluctuations were unveiled regarding the nutritional, phytochemical and bioactivity (antioxidant and anthelmintic, for *C. mariscus*) aspects assessed, affording considerable amounts of information on how to exploit the plant's chemical and biological assets more effectively. For example, the year-round availability of *C. mariscus* coupled with its relatively stable anthelmintic effects, proves advantageous for extraction of bioactive metabolites or future sustainable anthelmintic control practices. In the end, optimal collection periods depend on species and final biotechnological application targeted. Still, it is worth evoking that wild plants are affected by their ecosystem dynamics, which is a living entity by itself, thus, results obtained represent photographs of each harvesting period.

Do in vivo trials sustain the interest for further veterinary applications?

Unfortunately, under the studied conditions, no. In Chapter V, mostly non-significant *in vivo* parasitological findings were observed for *C. mariscus* and *L. monopetalum* extracts, applied at a low single dose, discouraging phytotherapeutical applications. However, it is the author's belief that improving methodological aspects of the *in vivo* experimental design may lead to measurable therapeutic effects, as discussed. Future work may also consider exploring the *in vivo* effects of the *H. italicum picardii* extract, as well as the effects of the extracts on the overall immune and antioxidant status of the host, considering their high phenolic and antioxidant traits.

As initially projected, this thesis outcomes contributed to the scientific understanding on the chemical and biological features of Mediterranean salt-tolerant plants, broadening

their biotechnological applications to the veterinary parasitology field. In the end, novel insights were gained for the anthelmintic discovery pipeline, enriching the current available knowledge, and providing new angles for future research.

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