

Disclosing the Bioactive Metabolites Involved in the *in Vitro* Anthelmintic Effects of Mediterranean Salt-Tolerant Plants: A Combined Approach Using PVPP and HPLC-ESI-MSⁿ

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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. The aims of this study are 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*, *Limnium monopetalum*, *Cladium mariscus* and *Helychrisum italicum picardi* 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 PVPP, and non-treated and treated samples were further characterized by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MSⁿ). 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.

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 [1]. 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 [2, 3]. *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp and *Nematodirus* spp. are the major relevant GIN species in Europe [4]. 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 (AAD) [5]. However, resistances to the different drug families are nowadays reported worldwide against different GIN species in different ruminants' species [6]. There are 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 [7]. 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. [8, 9] and sainfoin (*Onybrichis viciifolia* Scop.) [10], 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 [7]. So far, several plant extracts, fractions and individual compounds have been studied for their potential anthelmintic properties [11, 12]. 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 [12].

A wide number of extremophile plants, including salt-tolerant species, occur in the Mediterranean area [13]. 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 [14]. Additionally, former investigations reveal that many species exhibit relevant bioactive properties, like antioxidant, anti-inflammatory, and enzyme inhibitory activities [15] with diverse applications, including in veterinary medicine. Moreover, some species have ethnoveterinary uses [16], as for example, *Pistacia lentiscus* L., which is used as antiparasitic, for the treatment of bloat, constipation and dermatological ailments in sheep and goats [17]. 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* and 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-MSⁿ) of selected extracts treated or not with poly-(poly)vinylpolirrilodine (PVPP), a polyphenol-binding agent.

2. Material And Methods

2.1. Plant collection and processing

Plant species were selected based on the following criteria: 1) ethnopharmacological uses; 2) phenolic content reported in the literature; 3) availability/accessibility of the biomass and/or 4) unreported anthelmintic properties. Thus, aerial parts of *Pistacia lentiscus* L. (Anacardiaceae), *Cladium mariscus* (L.) Pohl (Cyperaceae), *Inula crithmoides* L. (Asteraceae), *Helichrysum italicum* (Roth) G. Don subsp. *picardi* (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) were collected in 4 districts of the Algarve coastal region (Southern Portugal), between 2017 and 2018 (Table 1). *Inula crithmoides*, *C. soldanella*, *M. marina*, *P. coronopus* and *L. monopetalum* are halophyte plants included in the eHALOPH database [18] while others such as *P. lentiscus*, *C. mariscus* and *C. maritima* have recognized salt-tolerance despite not yet included in eHALOPH database. After collection, samples were taken to the laboratory, washed, frozen

at -20°C, freeze-dried (Lyofalfa 15) for three days, and grinded using a coffee and a ball miller (Retsch PM 100).

Table 1
Plant collection details, including collected organs, date, location and voucher number.

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

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 identified by Dr. Luísa Custódio (CCMAR) and kept in the XtremeBio group herbarium, at Centre of Marine Sciences (CCMAR), University of Algarve (UALg), Faro, Portugal (Table 1).

2.2. Sample preparation

Dried ground samples were extracted with an 80:20 % acetone:water solution (1:40, w/v), as previously used for the successful extraction of phenolic compounds and tannins from different salt-tolerant species [19], at room temperature (RT) for 16h, 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.

2.3. Phenolic content of the extracts

2.3.1. Total Phenolic Content (TPC)

The TPC of the extracts was estimated using the Folin-Ciocalteu (F-C) reagent [20]. 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 RT, 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, at RT, 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)).

2.3.2. Total Flavonoid Content (TFC)

TFC was determined by the aluminum chloride (AlCl₃) method [21], by mixing 50 µL of the extracts at 10 mg mL⁻¹ with 50 µL of 2% AlCl₃ in methanol and left to incubate for 10 min at RT. 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).

2.3.3. Condensed Tannins Content (CTC)

CTC was evaluated by the 4-dimethylaminocinnamaldehyde-hydrochloric acid (DMACA-HCl) colorimetric method [22] adapted to 96-well microplates [23]. Ten µL 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).

2.3.4. Chemical profiling by high-performance liquid chromatography with electrospray ionization mass spectrometric detection (HPLC-ESI-MSⁿ)

HPLC-ESI-MSⁿ 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 C₁₈ analytical column (150 x 3.0 mm; 5 µm particle size) with a Polar C₁₈ Security Guard cartridge (4 x 3.0 mm), both purchased from Phenomenex. Detailed chromatographic conditions are available in Supplementary Material files. Compound's identification was performed by mass spectrometry data. Compound's 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). Calibration graphs were constructed in the 0.5–100 mg/mL range. 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.

2.4. In vitro *anthelmintic* assays

2.4.1. *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 hours after collection [24]

2.4.2. LEIA

LEIA was performed as previously described by Bahuaud and colleagues [25]. The extracts were prepared in serial concentrations in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.05 M NaCl, pH 7.2) and incubated with L3 larvae (approx. 800 larvae/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 were 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 exsheathment 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 (400x). 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) x 100].

2.4.3. 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/well) and exposed to the extracts at concentrations ranging from 5000 to $78 \mu\text{g mL}^{-1}$ in PBS. Plates were incubated at 27°C for 48h 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: $\%EH = [(\text{number of larvae}) / (\text{number of eggs} + \text{larvae}) \times 100]$.

2.5. Polyvinylpolypyrrolidone (PVPP) treatment

PVPP is a polyphenol inhibitor that binds to tannins and flavonoids, removing these metabolites from the solution [26]. 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 at concentrations of $2500 \mu\text{g mL}^{-1}$ and $1200 \mu\text{g mL}^{-1}$, respectively for eggs and larvae assays, in PBS, and incubated overnight at 4°C . The maximum concentration tested for LEIA was $1200 \mu\text{g mL}^{-1}$; for EHIA it was $2500 \mu\text{g mL}^{-1}$. Thereafter, the samples were centrifuged for 10 min at 4500 rpm and the supernatant depleted in polyphenols were tested in LEIA and EHIA assays. The extracts exposed or not to PVPP plus a negative control, PBS were run in parallel.

2.6. Statistical analyses

At least four replicates per plant and per concentration were included in all experiments. The results on phenolic content are expressed as mean $\pm$ standard error of the mean (SEM). Anthelmintic data are expressed as the concentrations inhibiting 50% of larval exsheathment or egg hatching (IC_{50} values, $\mu\text{g mL}^{-1}$) and 95% confidence intervals (CI). IC_{50} values and 95% CI were obtained by Probit analysis, and significant differences among IC_{50} values were detected using relative median potency estimates, using IBM SPSS Statistics v. 26.0 software. Spearman correlations were calculated between the total flavonoids, total phenols and the IC_{50} values for LEIA on the 2 nematode species.

3. Results And Discussion

3.1. Total Phenolics, Total Flavonoids and Condensed Tannins contents

The phenolic contents of the extracts are presented in Table 2. The phenolic content of the extracts is presented in Table 2. The total phenolic content of all species ranged between $14.2\text{--}226.3 \text{ mg GAE Eq. g}^{-1}$ DW extract while the total flavonoid content of ranged between 13.3 and $45.4 \text{ mg QE g}^{-1}$ DW. Lopes and colleagues (2016) reported higher TPC values for *C. mariscus* ($254 \text{ mg GAE g}^{-1}$ DW), *C. soldanella* ($144 \text{ mg GAE g}^{-1}$ DW), *I. crithmoides* ($141 \text{ mg GAE g}^{-1}$ DW), *L. monopetalum* ($248 \text{ mg GAE g}^{-1}$ DW) 80% acetone water extracts, except for *P. lentiscus* (130 mg g^{-1} DW), but lower flavonoid contents in comparison to our work ($1.26\text{--}13.8 \text{ mg rutin g}^{-1}$ DW) [15]. In another work, *H. italicum picardi* infusions

and decoctions of aerial organs have been previously described as rich sources of flavonoids (91.8–119 mg rutin 200 mL⁻¹) [27]. Moreover, a lower combined TPC values 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) [28]. 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) [15]. It is worth to mention 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 2
Phenolic content of acetone water extracts of selected plant species.

Species	TPC	TFC	CTC
<i>Helichrysum italicum</i> subsp. <i>picardi</i>	83.7 ± 0.6	45.4 ± 1.3	n.d.
<i>Inula crithmoides</i>	27.2 ± 1.1	13.3 ± 0.1	n.d.
<i>Pistacia lentiscus</i>	226.3 ± 0.8	28.9 ± 0.4	607.3 ± 29.4
<i>Calystegia soldanella</i>	73.2 ± 0.8	42.0 ± 1.0	n.d.
<i>Cladium mariscus</i>	112.3 ± 2.1*	18.5 ± 0.4*	153.1 ± 2.2*
<i>Medicago marina</i>	14.2 ± 0.5	27.0 ± 0.8	n.d.
<i>Plantago coronopus</i>	160.0 ± 3.0	25.2 ± 0.5	n.d.
<i>Limoniastrum monopetalum</i>	96.7 ± 2.9	16.0 ± 0.3	281.4 ± 22.6
<i>Crucianella maritima</i>	25.5 ± 0.9	20.4 ± 0.2	n.d.
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 [49].			

3.2. In vitro anthelmintic properties

Table 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 (IC₅₀ = 27.8–29.7 µg mL⁻¹) and egg hatching processes (IC₅₀ = 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 [29–32]. In previous studies, *P. lentiscus* extracts (acetone, ethanol and/or water) exhibited less than 20% larvae exsheathment and migration at 1200 µg mL⁻¹ [29, 31]. Nevertheless, the results for the *in vitro* egg hatching assay are herein, to the best of our knowledge, described for the first time.

Table 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 IC₅₀ values (µg mL⁻¹) and 95% confidence intervals in brackets.

Species	LEIA		EHIA	
	<i>H. contortus</i>	<i>T. colubriformis</i>	<i>H. contortus</i>	<i>T. colubriformis</i>
<i>Helichrysum italicum</i> subsp. <i>picardi</i>	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)
<i>Inula crithmoides</i>	300.8 ^{Ac} (231.5-391.2)	1030.8 ^{Be} (731.3-1563.0)	n.d.	n.d.
<i>Pistacia lentiscus</i>	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)
<i>Calystegia soldanella</i>	270.6 ^{Ac} (204.9-368.2)	270.8 ^{Ad} (197.7-384.4)	n.d.	n.d.
<i>Cladium mariscus</i>	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)
<i>Medicago marina</i>	222.6 ^{Ac} (179.8-278.6)	211.2 ^{Ad} (159.7-282.2)	n.d.	3860.5 ^d (3501.6-4343.8)
<i>Plantago coronopus</i>	94.0 ^{Ab} (71.6-121.2)	212.4 ^{Bd} (156.3-292.6)	n.d.	n.d.
<i>Limoniastrum monopetalum</i>	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)
<i>Crucianella maritima</i>	447.2 ^{Ad} (302.5-707.7)	1024.5 ^{Be} (616.9-2153.1)	n.d.	n.d.

n.d., not determined since IC₅₀ 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.

Following *P. lentiscus*, *L. monopetalum*, *C. mariscus* and *H. italicum. picardi* extracts exhibited the most promising results towards both GIN species and life stages (Table 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 $50 \mu\text{g mL}^{-1}$ (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 $2102.5 \mu\text{g mL}^{-1}$, respectively). *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 ($IC_{50} = 77.8\text{--}88.9 \mu\text{g mL}^{-1}$), without significant differences between both parasite species ($p > 0.05$). In contrast, in the EHIA, *C. mariscus* was more effective towards *H. contortus* ($IC_{50} = 1496.6 \mu\text{g mL}^{-1}$) than *T. colubriformis* ($IC_{50} = 2575.5 \mu\text{g mL}^{-1}$; $p < 0.05$). *Helichrysum italicum* subsp. *picardi* (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 and $132.5 \mu\text{g mL}^{-1}$ on LEIA, and 2947.7 and $3707.5 \mu\text{g mL}^{-1}$ on EHIA. Interestingly, *H. contortus* larvae and eggs were more susceptible to the *H. italicum picardi* 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 [7]. Moreover, these metabolites have different effects, depending on the target parasite species and life development stages [7]. A higher susceptibility of *H. contortus* in comparison to *T. colubriformis*, as observed for *C. mariscus* and *H. italicum picardi* extracts, has been previously documented for other bioactive plants and individual chemical structures, such as sainfoin, and depending on the ratios of prodelphinidins/procyanidins [10, 33, 34]. The authors suggest that such differences can reflect dissimilarities on the composition of specific parasite sheath proteins, that interact differently with the chemical groups [33, 34]. 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 [7, 35]. This may explain the results obtained for *P. coronopus*, which was more active against larvae exsheathment ($IC_{50} = 94.0$ and $212.4 \mu\text{g mL}^{-1}$), 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 [36, 37].

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 saltmarshes. These four species were mildly to poorly active on both assays (Table 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 [32], suggesting significant chemical diversity among the genus.

Overall, the nine plant extracts had comparable effects between the two GIN species (Spearman 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 (Spearman correlation; $R^2 = 0.783$; $p < 0.05$), suggesting that these metabolites may be involved in the anti-parasitic nematode's effects.

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 [26]. 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. 1 and 2, respectively. The application of all the extracts with PVPP largely restored the egg hatching process (Fig. 1) 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 [38]. 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. 2): the application of the *L. monopetalum* extract, resulted in 60–70% of larvae exsheathment of both parasite species after PVPP addition for 60 minutes, in contrast to 0% in the non-treated sample; the extract from *H. italicum picardi* pre-incubated with PVPP remained mostly completely active. Subtle changes were observed for *C. mariscus* (approx. 20–40 % of larvae exsheathment after 60 minutes 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 picardi*, *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 [29].

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 it is 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 [29]. 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 [29, 30]. 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 [39]. In an attempt to address these scientific questions, and elucidate the possible metabolites involved, we have conducted an HPLC-ESI-MSⁿ comparative analysis on the active samples, before and after PVPP treatment.

3.4. HPLC-ESI-MSⁿ comparative analysis of the chemical profile of non-treated and treated-PVPP samples

The HPLC-ESI-MSⁿ analysis was performed in the most active extracts, with and without PVPP. Obtained chromatograms are represented in Fig. 3 while the chemical profile is depicted in Tables 4–7. The characterization of the compounds is detailed in Supplementary Material files.

Table 4

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

No.	tR (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) MS3 [495→343]: 191 (99), 169 (100), 125 (20) MS4 [495→343→169]: 125 (100)	di- <i>O</i> -Galloylquinic acid	
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) MS3 [495→343]: 191 (100), 169 (77), 125 (10)	di- <i>O</i> -Galloylquinic acid	
14	7.8	495	MS2 [495]: 343 (100), 325 (36), 191 (12), 169 (15) MS3 [495→343]: 191 (40), 173 (9), 169 (100), 125 (10)	di- <i>O</i> -Galloylquinic acid	
15	8.4	183	MS2 [183]: 168 (100) MS3 [183→168]: 124 (100)	Methyl gallate	
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) MS3 [457→169]: 125 (100)	(Epi)gallocatechin gallate	
34	15.5	631	MS2 [631]: 479 (100) MS3 [631→479]: 317 (100), 316 (93), 179 (10) MS4 [631→479→317]: 271 (100), 179 (38)	Myricetin-hexoside-gallate	
38	16.6	625	MS2 [625]: 317 (100), 316 (87) MS3 [625→317]: 271 (100), 179 (90), 151 (22)	Myricetin- <i>O</i> -rutinoside	+

No.	tR (min)	[M-H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
41	17.1	493	MS2 [493]: 317 (100) MS3 [493→317]: 179 (100), 151 (29)	Myricetin-O-glucuronide	
43	17.5	479	MS2 [479]: 317 (100), 316 (97) MS3 [479→317]: 271 (100), 179 (66), 151 (12)	Myricetin-O-hexoside	
45	18.8	615	MS2 [615]: 463 (100), 301 (42) MS3 [615→463]: 301 (100) MS4 [615→463→301]: 179 (98), 151 (100)	Quercetin-hexoside-gallate	
47	19.6	449	MS2 [449]: 317 (44), 316 (100) MS3 [449→316]: 271 (100), 179 (26)	Myricetin-O-pentoside	
49	20.1	463	MS2 [463]: 317 (95), 316 (100) MS3 [463→316]: 271 (100), 179 (80), 151 (20)	Myricetin-O-deoxyhexoside	+
51	20.9	463	MS2 [463]: 301 (100) MS3 [463→301]: 179 (100), 151 (50)	Quercetin-O-hexoside	
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) MS3 [433→301]: 271 (100), 179 (87), 151 (68)	Quercetin-O-pentoside	
62	23.5	447	MS2 [447]: 285 (100) MS3 [447→285]: 255 (100), 229 (37), 227 (33)	Kaempferol-O-hexoside	
66	24.8	447	MS2 [447]: 301 (100) MS3 [447→301]: 179 (48), 151 (100)	Quercetin-O-deoxyhexoside	+
70	26.7	585	MS2 [585]: 301 (100) MS3 [585→301]: 179 (100), 151 (98)	Quercetin-pentoside-gallate	

No.	tR (min)	[M- H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
73	29.0	431	MS2 [431]: 285 (100) MS3 [431→285]: 257 (93), 255 (100), 241 (55), 229 (36)	Kaempferol-O-deoxyhexoside	+
74	30.2	569	MS2 [569]: 285 (100) MS3 [569→285]: 285 (100), 257 (37), 151 (86)	Kaempferol-pentoside-gallate	
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	+

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 4; Suppl. files, Table I). In agreement to 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 [40]. 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 complex structures (gallotannins and ellagitannins) [41]. Plant extracts containing hydrolysable tannins with gallic acid units were more effective as anthelmintics than those containing condensed tannins [42]. Nevertheless, the oligomerization and molecular weight of tannins may affect the anthelmintic activity, as is the case, for example, of ellagitannins and condensed tannins [34, 43]. 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. colubriformis* egg hatching (20% vs 100% inhibition at 1mg mL⁻¹), and also more effective on immobilizing infective larvae (100% inhibition at 100–150 µg mL⁻¹) [44].

In *P. lentiscus* PVPP-treated samples, the concentration of flavonoid glycosides (0.17 mg g⁻¹ DW) and galloquinic acid (2.2 mg g⁻¹ DW) drastically dropped (Suppl. files, Table I), 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 picardi* extract ($150 \text{ mg g}^{-1} \text{ DW}$), followed by quercetin-*O*-glucosides (approx. $31 \text{ mg g}^{-1} \text{ DW}$; Table 5; Suppl. files, Table II). These findings were expected, since previous works identified high contents of these metabolites in aerial organs of the same species [27, 45]. 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* [46]. Additionally, chlorogenic acid exhibited an IC_{50} value of $92.4 \text{ } \mu\text{g mL}^{-1}$ against L3 larvae exsheathment of *H. contortus* and was also effective on preventing larvae hatching from eggs ($\text{IC}_{50} = 520.8 \text{ } \mu\text{g mL}^{-1}$) [47]. These results point out to the potential of caffeoylquinic and dicaffeoylquinic acids to be the active metabolites of *H. italicum picardi* 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 $1200 \text{ } \mu\text{g mL}^{-1}$ [48].

Table 5

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

No.	t_R (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- <i>O</i> -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- <i>O</i> -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- <i>O</i> -hexoside	

No.	t_R (min)	[M-H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
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- <i>O</i> -hexoside	
54	21.6	493	MS ² [493]: 331 (100) MS ³ [493→331]: 316 (100)	Mearnsetin- <i>O</i> -hexoside	
56	22.2	477	MS ² [477]: 315 (100), 314 (16) MS ³ [477→315]: 300 (100)	Isorhamnetin- <i>O</i> -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- <i>O</i> -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- <i>O</i> -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- <i>O</i> -hexoside	

No.	t_R (min)	[M-H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
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- <i>O</i> -deoxyhexoside- <i>O</i> -hexoside	

In *H. italicum picardi* 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 picardi* treated with PVPP on larvae exsheathment is most likely due to the high content of chlorogenic acid remaining in the sample [47]. 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) [46].

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 [15, 49]. 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 6; Suppl. files, Table III). Flavan-3-ols and proanthocyanidins have recognized anthelmintic effects [44, 50], and therefore, they 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 (IC₅₀ = 17.1 and < 71.5 μM) [51]. Interestingly, Klongsiriwet and colleagues (2015) found that luteolin, even at low concentrations (30 μM), display synergistic effects with procyanidins, leading to a 5-fold lower IC₅₀ of the mixture in comparison to the procyanidin fraction alone (75.9 vs 356 μg mL⁻¹) [51]. 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 6; Suppl. files, Table III). As previously addressed, chlorogenic acid exhibits significant

anthelmintic activity *in vitro* against *H. contortus* larvae exsheathment and egg hatching [47]. 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 [52] and two flavone-*C*-glycosides namely isoschaftoside and schaftoside shown strong toxicity ($LC_{50} = 114.66 \mu\text{g/mL}$ and $323.09 \mu\text{g/mL}$) against the plant parasitic nematode *Meloidogyne incognita* [53]. One can speculate that the partial remaining activity of *C. mariscus* extract on L3 larvae exsheathment may be accounted to chlorogenic acid, but the possibility of *C*-glycosyl flavones are active should not be excluded. Moreover, it is worth noticing that compound **20** and **39** are still unidentified, although present in PVPP-treated samples.

Table 6

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

No.	t_R (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	

No.	t_R (min)	[M-H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
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- <i>C</i> -hexoside- <i>C</i> -pentoside	+
35	15.9	563	MS ² [563]: 545 (14), 503 (15), 473 (48), 443 (100), 383 (37), 353 (43)	Apigenin- <i>C</i> -hexoside- <i>C</i> -pentoside	+
37	16.5	447	MS ² [447]: 429 (14), 357 (70), 327 (100), 285 (3)	Luteolin-6- <i>C</i> -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- <i>C</i> -pentosyl-8- <i>C</i> -pentoside	+
42	17.3	563	MS ² [563]: 503 (22), 473 (100), 443 (69), 383 (61), 353 (97)	Apigenin- <i>C</i> -hexoside- <i>C</i> -pentoside	+
53	21.4	447	MS ² [447]: 285 (100) MS ³ [447→285]: 285 (100), 241 (47), 151 (10)	Kaempferol- <i>O</i> -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- <i>C</i> -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 7

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

No.	t_R (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 (3), 143 (14), 119 (24), 113 (6)	Disaccharide (HCl adduct)	+
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) MS ³ [303→223]: 208 (100), 179 (37), 164 (35), 149 (5)	Sinapic acid sulfate	+
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) MS ³ [457→169]: 125 (100)	Gallic acid derivative	+
33	14.4	457	MS ² [457]: 329 (100), 245 (26), 203 (23), 165 (24) MS ³ [457→329]: 314 (100)	Unknown	+
46	19.1	252	MS ² [252]: 212 (100), 204 (4)	Unknown	
48	19.8	609	MS ² [609]: 301 (100) MS ³ [609→301]: 179 (100), 151 (78)	Rutin	
52	21.2	477	MS ² [477]: 301 (100) MS ³ [477→301]: 179 (90), 151 (100)	Quercetin- <i>O</i> -glucuronide	

No.	t_R (min)	[M-H] ⁻ m/z	m/z (% base peak)	Assigned identification	PVPP
55	21.7	567	MS ² [567]: 331 (100) MS ³ [567→331]: 316 (100), 179 (67), 151 (33)	Unknown	
64	24.1	437	MS ² [437]: 357 (100), 151 (52) MS ³ [437→357]: 342(5), 311 (6), 151 (100), 136 (24)	Pinoresinol	+
65	24.4	395	MS ² [395]: 315 (100) MS ³ [395→315]: 300 (100), 271 (8), 255 (13)	Isorhamnetin sulfate	
67	25.2	425	MS ² [425]: 345 (100) MS ³ [425→345]: 330 (100), 315 (34) MS ⁴ [425→345→330]: 315 (100), 285 (74)	Methylated flavonoid sulfate	
71	27.2	425	MS ² [425]: 345 (100), 330 (15) MS ³ [425→345]: 330 (100) MS ⁴ [425→345→330]: 315 (100), 271 (10)	Methylated flavonoid sulfate	
76	32.5	439	MS ² [439]: 359 (100) MS ³ [439→359]: 344 (100) MS ⁴ [439→359→344]: 329 (100)	Methylated flavonoid sulfate	+
80	36.9	439	MS ² [439]: 359 (100) MS ³ [439→359]: 344 (100), 329 (18)	Methylated flavonoid sulfate	
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	+

Previous works identified several phenolic compounds in *L. monopetalum* extracts including gallic, vanilic, ferulic, syringic, p-hydroxybenzoic, protocatechuic, chlorogenic and trans-cinnamic acids, and also quercetin, apigenin, amentoflavone, flavones, methyl gallate and myricetin [54, 55]. 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 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 [56, 57]. 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 [58] and *Halimione portucaloides* (L.) Aellen [59]. The pharmacological interest on 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 [60].

Besides epigallocatechin ($9.46 \text{ mg g}^{-1} \text{ DW}$), the concentration of isorhamnetin sulfate (**65**) was high in *L. monopetalum* ($6.4 \text{ mg g}^{-1} \text{ DW}$) as well as phenolic acids and its derivatives ($10.3 \text{ mg mL}^{-1} \text{ DW}$; **7**, **16**, **24**, **32**). Delgado-Núñez and colleagues (2020) attributed the main anthelmintic effects of *Prosopis laevigata* Willd. M. Johnston to isorhamnetin, which caused 100% of mortality on *H. contortus* eggs at the lowest concentration tested ($700 \text{ } \mu\text{g mL}^{-1}$), being also highly effective towards larvae ($\text{IC}_{50} = 2.07 \text{ mg mL}^{-1}$) [61]. The glycoside isorhamnetin-3- rutinoside decreased *H. contortus* L3 migration in 35% at $120 \text{ } \mu\text{g mL}^{-1}$ [48]. 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 = $0.56\text{--}4.93 \text{ } \mu\text{g mL}^{-1}$) and larval development ($\text{IC}_{50} = 22\text{--}33 \text{ } \mu\text{g mL}^{-1}$) [62]. 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 shown a 2-fold increase in the larvicidal activity of rutin [62]. 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.

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 since the last 20 years [63]. 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. *picardi*, *I. crithmoides*, *C. soldanella*, *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* subsp. *picardi* were the most active against both parasite species and life stages (eggs and L3) targeted. The comparative HPLC-ESI-MSⁿ analysis coupled with 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 picardi*; 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*, which is a species widely investigated for its phytochemical content and anthelmintic effects. Moreover, it would be interesting to fully elucidate which of the main metabolites exert anthelmintic properties, either alone and/or in synergy, and provide information on some compounds structure-activity effects. Still, the results obtained in this study for *L. monopetalum*, *C. mariscus* and *H. italicum* subsp. *picardi* 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.

Declarations

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Author's contributions

MO performed the design of the study, collection and extraction of plant material, preparation of the extracts, *in vitro* assays and draft of the manuscript, CSL and SK assisted in the anthelmintic assays, EJLM performed the HPLC-ESI-MSⁿ analysis, HH performed the design of the study and review of the manuscript, LC performed the design of the study, review of the manuscript and final approval.

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Figures

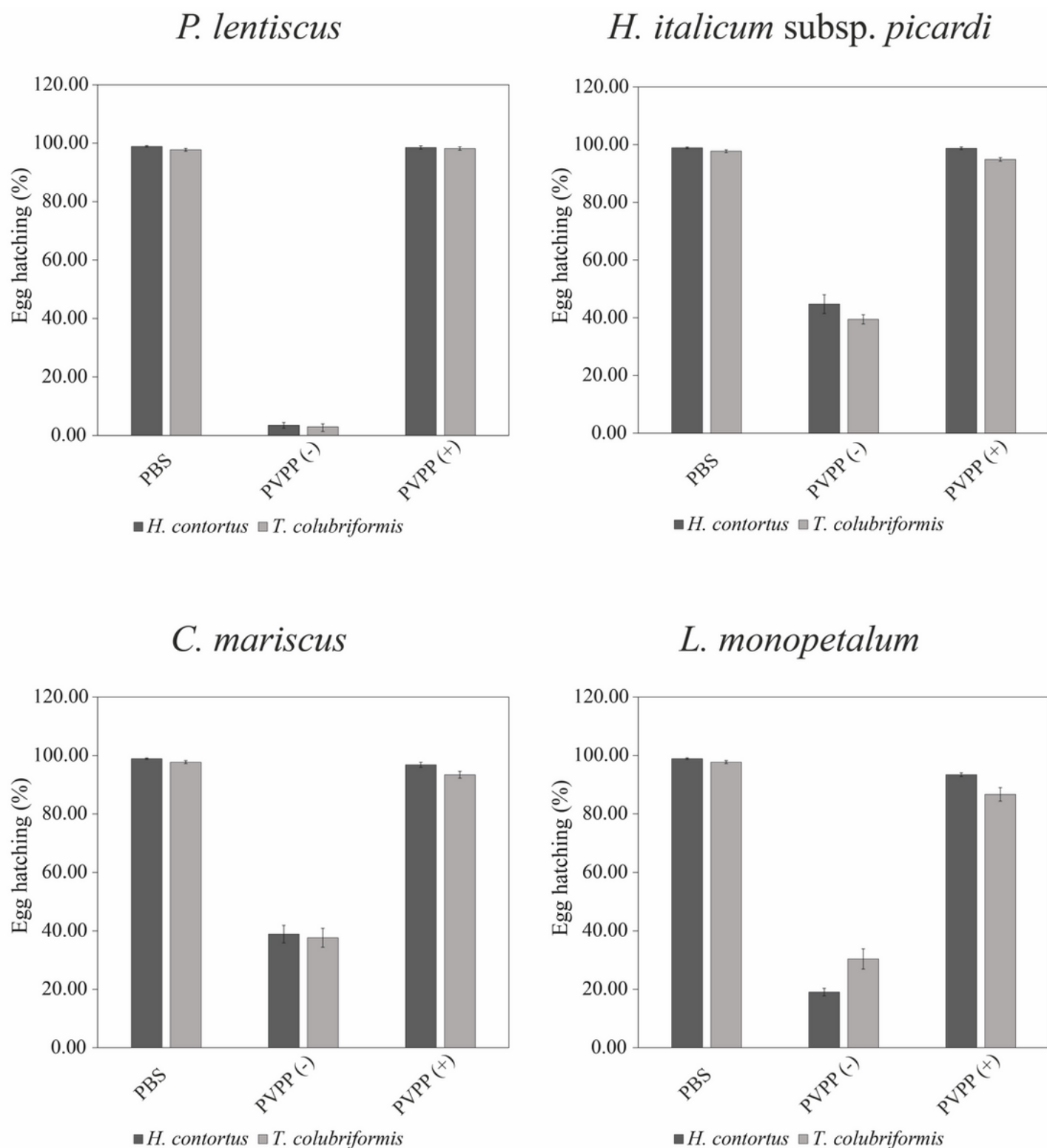


Figure 1

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 $\mu\text{g mL}^{-1}$, either treated [PVPP(+)] or not [PVPP(-)] with PVPP.

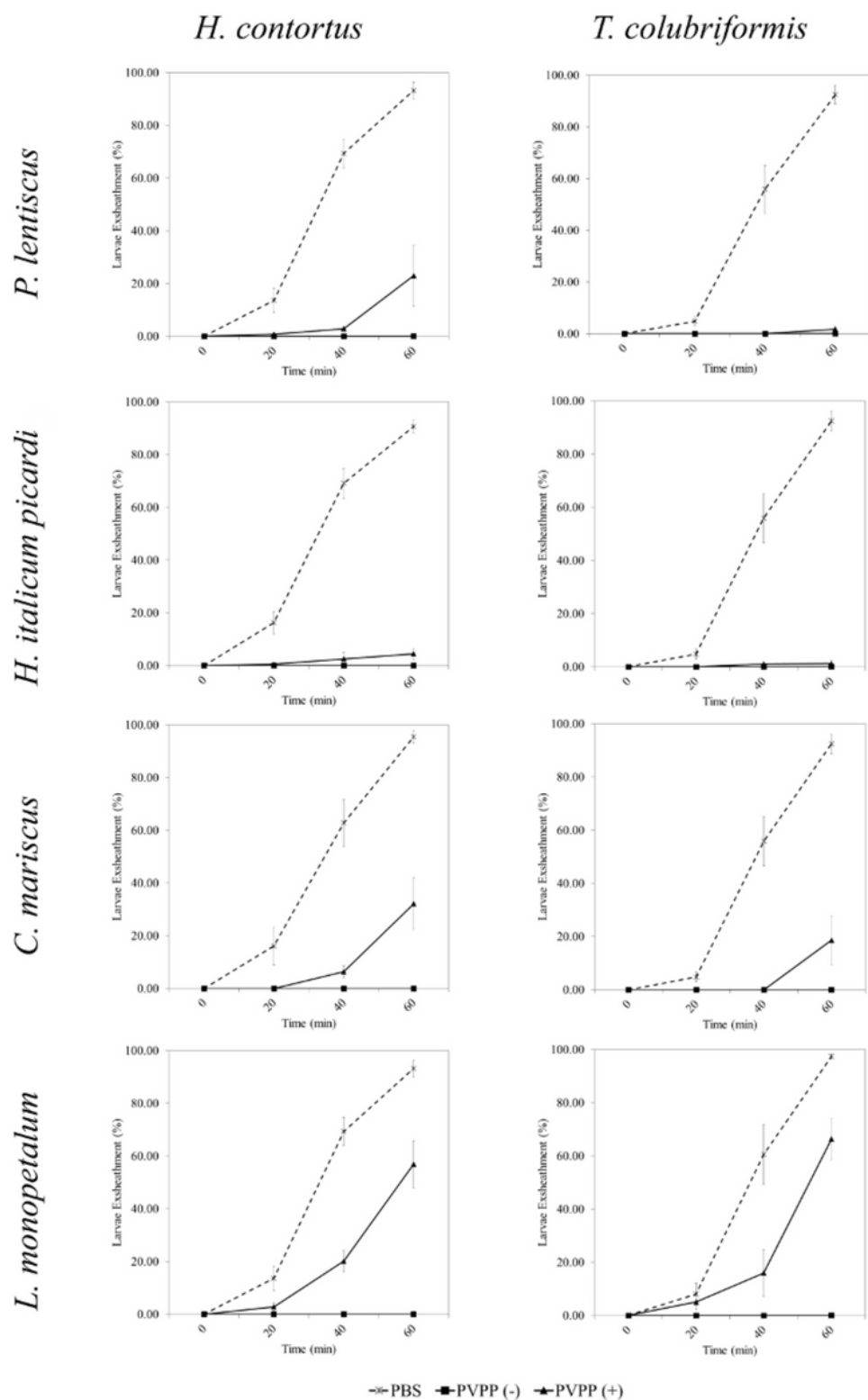


Figure 2

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.

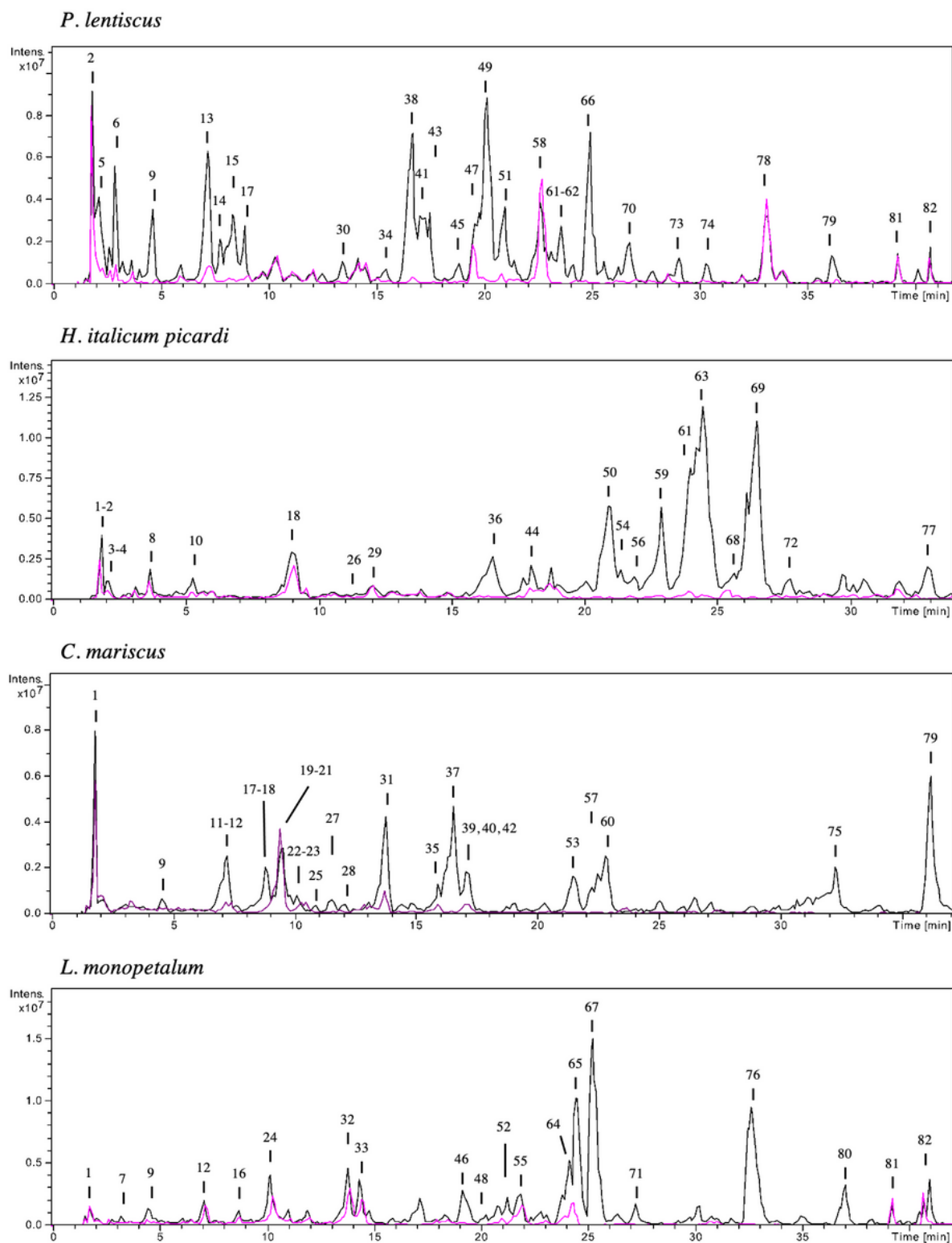


Figure 3

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 4 - 7.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Oliveiraetal.SupplementaryMaterialfiles52.docx](#)