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Comparative study of GC-MS characterization, antioxidant activity and hyaluronidase inhibition of different species of *Lavandula* and *Thymus* essential oils

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Abstract: The chemical compositions of essential oils of *Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula hybrida* cultivar Grosso and cultivar Super, *Thymus zygis* with high proportions of thymol and linalool and *Thymus hyemalis*, from Murcia country (Spain), were studied in relative (%), absolute (mM) and chiral concentrations by GC/MS. Hyaluronidase inhibition and antioxidant activities of the essential oils were evaluated using ABTS$^{\bullet+}$, DPPH$^{\bullet}$, ORAC, chelating power, hydroxyl radical, nitric oxide, TBARS and reducing power assays. Linalool and linalyl acetate were the most abundant components in the *Lavandula* genus whereas thymol, linalool and 1,8-cineole were the most abundant molecules in the respective *Thymus* species. Chiral determination of the main components showed (+)-enantiomers like terpinen-4-ol, β-pinene, borneol and α-terpineol and (−)-enantiomers like linalool, linalyl acetate and camphene in *Lavandula* sp. In the case of *Thymus* sp. (+)-enantiomers like α-pinene, limonene, terpinen-4-ol and α-terpineol and (−)-enantiomers like borneol were found. Essential oils containing thymol were found especially powerful in all assays but chelating power, ORAC and hydroxyl radical scavenging assays. The capacity for inhibiting hyaluronidase showed that *T. zygis* with a high proportion of thymol was the most effective inhibitor. Essential oils containing thymol and linalool/linalyl acetate have a potential use as antioxidant agents. Thymol shows strong inhibition of hyaluronidase.

Keywords: lavandula; thymus; antioxidant; hyaluronidase inhibition; essential oil

Introduction

The genus *Lavandula* belongs to the *Lamiaceae* family and includes 39 known species. However, there are three important species because of their terpenoid-rich essential oils (EOs), which are obtained by steam distillation. They are *L. angustifolia* Mill. (*L. officinalis* Chaix ex. Vill, *L. spica* L, *L. vera* DC) or true lavender; *L. latifolia* Medik. or spike lavender; and the natural hybrid *L. x intermedia* Emeric ex Loisel (*L. hybrida* L.) or lavandin that is derived from a cross of *L. latifolia* × *L. angustifolia*. EOs are extensively used in perfumes, in cosmetics, in food manufacturing for flavouring beverages, ice creams, candies, baked goods and chewing gums, and in aromatherapy as relaxants.[1,2]

Lavandula angustifolia is one of the most desired lavender oils in the cosmetic and aromatherapeutic industries, which is as a result of the high concentration of both linalool/linalyl acetate and low-camphor concentration. However, true lavender produces this interesting EO in relatively low amounts. For the higher production of this EO, hot, dry climates and medium altitudes (700–1200 m) are required.[3] The yield of EO from lavandin is three-fold higher than the one of *L. angustifolia*, albeit with much lower application in perfumery and therapy owing to the undesirably high levels of camphor. Such oil is preferentially used as the antiseptic, antifungal and antibacterial agent.[3] These biological properties have also been attributed to *L. angustifolia* and *L. latifolia*. In addition, they have also been used as sedative, carminative, anti-depressive and anti-inflammatory agents, although clinical studies show inconclusive results.[4] *Lavandula latifolia* is also believed to be effective for burns and insect bites.[5]

The genus *Thymus* is one of the eight most important genera regarding the number of species included: more than 300, including hybrids, varieties and ecotypes.[6]

Thymus hyemalis Lange, winter thyme, can be found mainly in the South East of Spain (Alicante, Murcia and Almeria).[7] Chemical variability has been reported for the essential oils of this species, the presence of at least four chemotypes in that region of Spain was stated beyond the seasonal variations and edaphic and climatic conditions.[7,8]

The antimicrobial activity of *T. hyemalis* EO was found in 2008,[9] being thymol chemotype more active than carvacrol chemotype. The antimicrobial activity along with antioxidant activity of winter thyme EO were also reported.[10]

Thymus zygis Loefl. ex L. is a widespread endemic plant in the Iberian Peninsula. At least eight chemotypes have been found in

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this region.[11] Seasonal variations and phenological stages are also factors that contribute to the chemical variability of *T. zygis* EOs.[12]

Antimicrobial, antigiardial, antiviral, anti-enzymatic and antioxidant activities of *T. zygis* EOs have been reported.[10,13–18]

Gas chromatography (GC), coupled with mass spectrometry detection, is a powerful technique used for volatile components analysis, as it provides qualitative and quantitative data for complex mixtures such as those usually present in EOs.[19]

The quantitative composition of EOs may be estimated using relative percentage abundance, internal standard normalized percentage abundance and true quantitation of one or more compounds by a validated method.[20] So far, relative percentage abundance is the most commonly method used in the EO analysis. However, it should only be used to measure relative component ratios in a single sample and not to compare compositions of a group of EOs, because relative percentage abundances are not standardized.[20]

Furthermore, there are scarce chiral studies accounting for the main components of the studied EOs. Observing the enantiomeric distribution of the main molecules of the EOs is highly interesting, as different bioactivities and organoleptic properties depend on the enantiomeric ratios. These characteristics are useful in the formulation and authenticity assessment of drugs, fragrances and flavours.[1,21]

The antioxidant activities of the mentioned EOs have been found to be interesting because oxidation, induced by reactive oxygen and nitrogen species (ROS and RNS), can damage membranes, lipids and lipoproteins and can induce DNA mutation. These types of cell or tissue injuries have been associated with ageing, atherosclerosis, carcinogenesis and cardiovascular as well as Alzheimer's and other neurological diseases. Thus, preventing or minimizing these oxidation processes, by the use of antioxidant substances that scavenge hydroxyl ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) or other free radicals, may help in the treatment of the aforementioned illnesses.[1,4,6,22–24]

The extracellular matrix (ECM) breakdown is related with oxidative stress and degradation of proteins such as collagen and elastin. It is also related to hydrolysis, catalyzed by hyaluronidase, of proteoglycans with glycosaminoglycans such as hyaluronic acid. Hyaluronic acid is a polysaccharide composed of D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds, with a size from 5000 Da to 20 MDa *in vivo*. Hyaluronic acid imbibes water, provides resilience to cartilages, replaces fibres of degraded collagen and enhances the regeneration of collagen by the dermis and the ECM. The hyaluronic acid/hyaluronidase system also participates in many pathophysiological conditions such as envenomation, acrosome reaction/ovum fertilization, microbial pathogenesis and cancer progression. The inhibitors of hyaluronidase might serve as a contraceptive, anti-venom/toxin, anti-microbial, anti-aging, anti-inflammatory and anticancer agents.[25]

The aim of this study was to determine thoroughly the chemical composition and bioactivities of the EOs of four species of *Lavandula* genus (*Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula hybrida* cultivar Grosso and cultivar Super) and three of *Thymus* genus (two samples of *Thymus zygis* and one sample *Thymus hyemalis*). Source plants were grown under organic farming in the Murcia country (Spain). Their relative and absolute concentrations, as well as the proportions of their main chiral compounds, will be determined. Also, several antioxidant methods will evaluate the antioxidant capacity of EOs against different oxidant agents. Furthermore, the potential bioactivity of EOs on the important and multifunctional hyaluronic acid/hyaluronidase system will be determined. Thus, this study is focused on increasing the diversity of available compositions and bioactivities of these EOs with samples from Murcia and comparing them with EOs from different parts of the world. Hence, this study aims to broaden the knowledge about composition, antioxidant and anti-hyaluronidase properties of *Lavandula* and *Thymus* EOs.

Materials and methods

Plant material and extraction

Seven samples (300 g each) of *L. angustifolia*, *L. latifolia*, *L. hybrida* cultivar Grosso, *L. hybrida* cultivar Super, *T. zygis* with a high proportion of thymol, *T. zygis* with a high proportion of linalool and *T. hyemalis* (with identification numbers of the voucher specimens BMBA130901, BMBA130902, BMBA130903, BMBA130904, BMBA130905, BMBA130906 and BMBA130907, respectively) were obtained from organic farming fields dedicated to growing plants for industrial extraction of EOs through steam distillation, located in 'Region de Murcia', a country of the South East of Spain with a rich plant biodiversity. The species were identified and authenticated by Dr Pedro Sanchez-Gomez, Professor of Botany in the Plant Biology department of Murcia University and voucher specimens were prepared and deposited in the herbarium of the Department of Biochemistry and Molecular Biology-A.

The dry plants of each sample were separately subjected to steam distillation for 3 h with a Clevenger apparatus. The oil collected was dried over anhydrous sodium sulphate, obtaining strong smelling pale yellow oil that was stored at −20 °C until used.

Chemicals

The following compounds were purchased from Sigma-Aldrich-Fluka: pure compounds used as standard substances for GC identification, ABTS, DPPH, AAPH, fluorescein, PBS, 2-deoxyribose, sodium hydroxide, sodium borate, calcium chloride, bovine hyaluronidase, p-dimethylamino benzaldehyde, ferrozine, iron(II) chloride, iron(III) chloride, potassium hexacyanoferrate (II), potassium chloride, sodium dodecyl sulfate, n-butanol, BHT, mannitol, rutin, ascorbic acid. Analytic grade solvents, hydrogen peroxide, thiobarbituric acid and buffers were acquired from Merck. Sodium persulfate, Trolox, sodium hyaluronate and EDTA were acquired from Acros organics. Sodium nitroprusside was purchased from Riedel-deHaen. Griess reagent system kit was obtained from Promega. Trichloroacetic acid was obtained from VWR. Iron (II) sulfate was purchased from Panreac. Deionized laboratory water type I (18 MΩ · cm) was produced with a Millipore MilliQ-Reference device.

Fast gas chromatography-electron impact/mass spectrometry (FGC-EI/MS)

FGC-EI/MS analyses of the essential oils were conducted using an Agilent GC7890 chromatograph (Agilent Technologies, Santa Clara, CA, USA), coupled with an Agilent MS5975 mass spectrometer as a detector. The analysis was performed on a low bleed capillary fused-silica column, SLB-5 ms from Supelco (15 m length × 0.1 mm i.d. × 0.1 μm film thickness). The carrier gas used was hydrogen (flow rate constant: 0.8 ml/min, starting column head pressure: 46.345 psi), produced *ad hoc* with an electrolytic Parker-Domnik-Hunter generator, fed with type I laboratory water. The above-mentioned complex device was controlled by ChemStation software and analysed using ChemStation, MS-Search, AMDIS and the mass spectral databases NIST 08 and Wiley 7. The injector

temperature was held at 280 °C; the septum purge was set to 3 ml/min and the split valve was set to 100:1. The column temperature started at 60 °C and increased to 300 °C according to the temperature programme as follows: rate 20 °C/min to 142 °C, and rate 40 °C/min from 142 °C to 300 °C and then held for 0.5 min. MS conditions were as follows: temperature of the transfer line 280 °C, Electron Ionization energy 70 eV, mass range 30–300 atomic mass units, scan rate 21.035 scan/s. The EO compounds were identified based on retention time, retention index and mass spectra of the pure compounds (standards) compiled in an in-lab built library using MSD Chemstation Data Analysis. Tentative identification, for each component not available commercially, was attempted comparing the retention index and mass spectra against NIST and Wiley spectral databases using NIST MS Search 2.0 (NIST, Gaithersburg, MD, USA). The quantitative determination was carried out based on calibration curves of each of the commercially available components of the essential oils. Those curves were made using nonane (MS **43**, 57, 71, 85), tetradecane (MS 43, **57**, 71, 85) and hexadecane (MS 41, 43, **57**, 71) as internal standards, calibration ranges and suppliers for all substances are listed in Table 1. Calibration curves are expressed as the response ratio $\left(\frac{\text{analyte response}}{\text{internal standard response}}\right)$ vs. the concentration ratio $\left(\frac{\text{analyte concentration}}{\text{internal standard concentration}}\right)$, thus, correction factors of each analyte are embedded in the calibration curves. Each sample was evaluated in two different dilutions in isooctane, 1:10 and 1:1000 in order to determine exact concentrations of compounds.

The repeatability of five detections of 3-mM samples is shown in the RSD column for all standards. The limit of detection (LOD) was calculated by a signal-to-noise ratio of 3, and the limit of quantitation (LOQ) was determined by a signal-to-noise ratio of 10.[26]

In the case of non-commercially available compounds, the percentage of the peak area of the total ion chromatogram was evaluated. Retention indices (LRIs) were calculated using a homologous series of C_7-C_{30} n-alkanes standard solutions from Supelco, according to the IUPAC recommendations[27] for linear retention indices.

Enantioselective gas chromatography-electron impact/mass spectrometry (EsGC-EI/MS)

The same analytical device described above was used, this time equipped with an Astec Chiraldex B-DM column (30 m length × 0.25 mm i.d. x 0.12 μm film thickness) from Supelco. Performing under milder conditions, this column provided chiral chromatograms of the essential oils, where the chiral compounds were determined by the retention time of the pure enantiomers commercially available, and double checked with the NIST spectral database. The peak areas of the triplicates were integrated, and enantiomeric ratios of levorotatory (−) and dextrorotatory (+) enantiomers were determined. Conditions: injector temperature 200 °C, transfer line temperature 200 °C, split 100:1, temperature programme as follows: starting at 35 °C, the temperature increased to 170 °C at a rate of 4 °C/min, then the temperature decreased to 35 °C at a rate of 15 °C/min.

Determination of ABTS radical cation scavenging capacity

The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation decolourization assay was carried out using the method already reported in the literature[28] with slight modifications. An ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 3.5 mM ABTS with 2.45 mM potassium persulfate (final concentration) and keeping the mixture in the dark at room temperature for 12–16 h before use.

An ethanolic solution (10 μl) of the samples at various concentrations (0–0.5 g/l) was mixed with 990 μl of 0.035 mM $ABTS^{\bullet+}$ solution. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan). The ability to scavenge $ABTS^{\bullet+}$ was calculated using the formula given below:

$$\text{scavenging or inhibitory activity } (\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad (1)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample. Trolox was used as a reference to express the results in TEAC units (μmol Trolox equivalent / g essential oil).

Free radical scavenging activity (DPPH)

A methanolic stock solution (50 μl) of each sample at different concentrations was placed in a cuvette, adding 2 ml of 60 μM methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl).[29] Absorbance measurements were made at 517 nm, after 60 min of reaction in the dark, at room temperature. The values of scavenging activity were reported in TEAC units, according to the same process shown in the ABTS assay.

ORAC

The ORAC values account for the ability of the samples to scavenge the peroxyl radical (ROO·). ORAC values were determined using a slight modification of the reported method.[30]

This assay was performed at 37 °C for 60 min, in a Molecular Devices Gemini XPS fluorometer controlled by the software SoftMaxPro, using a 96-well plate, each well having a filled volume of 300 μl. Each sample was evaluated in triplicate using $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm. Each assay consisted of pH 7.5, 10 mM phosphate buffer, 1 μM fluorescein, 200 mM AAPH and an antioxidant sample in several concentrations. The organic solvent was 1% ethanol for all assays. The values of scavenging activity were reported in TEAC units, according to the same process shown in the ABTS assay.

Chelating power of metallic ions

The degree of chelation of ferrous ions by essential oils was evaluated according to the literature.[31] Briefly, samples were incubated 1:1 with 0.025 ml of 2 mM $FeCl_2$. The addition of 0.025 ml of 5 mM ferrozine initiated the reaction, and after 10 min, the absorbance at 562 nm was measured. An untreated sample served as the control. The percentage of chelating ability was determined according to Eqn (1). EDTA was used as a positive control.

Hydroxyl radical scavenging activity

The assay of hydroxyl radical ($OH^{\cdot}$) scavenging activity was developed with small modifications to the reported method.[32] Briefly, the reaction mixture was prepared with 10 mM $FeSO_4 7H_2O$, 10 mM EDTA, 10 mM 2-deoxyribose, 0.1 M phosphate buffer and sample at different concentrations in a test tube to give a total volume of 1.8 ml. Finally, 200 μl of 0.1% (v/v) H_2O_2 were added to the mixture and incubated at 37 °C for 4 h.

Table 1. Standards used in GC/MS and calibration curves for essential oils (EOs) analysis

Analyte	Calibration curve[a]	R^2	Calibration range (mM)	RSD (%)	LOD (mM)	LOQ (mM)	Standard source	Product Reference
Nonane	*Internal standard*						SAFC	442694
α-Thujene	y = 0.566x - 0.045	0.997	0.50 - 10.09	6.0	0.16	0.47	EML	
(−)-α-Pinene	y = 0.471x - 0.018	0.997	0.25 - 10.06	4.5	0.08	0.25	Fluka	80599
(+)-Camphene	y = 0.272x - 0.008	0.995	0.66 - 10.51	0.4	0.14	0.44	SAFC	w222909
Sabinene	y = 0.460x - 0.026	0.998	0.24 - 9.78	0.9	0.07	0.21	Extrasynthese	5062 S
(−)-β-Pinene	y = 0.400x - 0.016	0.995	0.26 - 10.29	3.7	0.09	0.26	Fluka	80609
3-Octanone	y = 0.309x - 0.031	0.996	2.56 - 10.24	4.1	0.64	1.95	SAFC	w280305
Myrcene	y = 0.188x - 0.024	0.993	2.24 - 8.97	3.5	0.56	1.70	Fluka	64643
Hexyl acetate	y = 0.452x - 0.057	0.992	2.40 - 9.59	1.7	0.32	0.96	Fluka	25539
α-Phellandrene	y = 0.430x - 0.012	0.998	0.50 - 9.52	0.9	0.16	0.49	Aldrich	77429
α-Terpinene	y = 0.425x - 0.035	0.997	0.45 - 9.45	2.8	0.15	0.44	Aldrich	86473
p-Cymene	y = 0.830x - 0.020	0.995	0.25 - 9.95	4.9	0.02	0.05	Aldrich	c121452
(+)-Limonene	y = 0.246x - 0.008	0.995	0.60 - 9.55	0.9	0.12	0.36	Fluka	62118
(*Z*)-β-Ocimene	y = 0.218x - 0.011	0.996	0.70 - 6.97	2.2	0.16	0.48	SAFC	w353901
1,8-Cineole	y = 0.233x - 0.008	0.995	2.39 - 9.55	8.7	0.60	1.82	SAFC	w246506
γ-Terpinene	y = 0.366x - 0.017	0.995	0.62 - 9.87	2.8	0.12	0.37	Aldrich	223190
(+)-*trans*-Sabinene hydrate	y = 0.268x - 0.007	0.996	0.63 - 10.00	2.1	0.18	0.56	Fluka	96573
(−)-Linalool	y = 0.214x - 0.008	0.994	0.88 - 8.80	4.9	0.22	0.67	Fluka	74856
1-Octen-3-yl acetate	y = 0.279x - 0.015	0.995	2.05 - 8.21	1.4	0.38	1.16	SAFC	w358207
(+)-Camphor	y = 0.181x - 0.024	0.994	0.99 - 9.85	1.5	0.25	0.76	Alfa Aesar	A10708
(−)-Borneol	y = 0.266x - 0.020	0.998	0.57 - 9.06	4.4	0.19	0.57	Alfa Aesar	A12684
(−)-Terpinen-4-ol	y = 0.241x - 0.003	0.997	0.60 - 9.57	4.3	0.20	0.60	Aldrich	11584
Hexyl butyrate	y = 0.332x - 0.034	0.991	4.84 - 7.74	0.6	0.77	2.34	SAFC	w256811
(+)-α-Terpineol	y = 0.175x - 0.003	0.998	1.02 - 10.23	4.5	0.26	0.79	Fluka	83073
Tetradecane	*Internal standard*						SAFC	442708
Verbenone	y = 0.183x - 0.032	0.994	1.30 - 9.88	1.8	0.42	1.28	Aldrich	218251
Citronellol	y = 0.137x - 0.014	0.998	0.90 - 8.69	9.0	0.29	0.87	SAFC	S60330
Methyl ether of carvacrol	y = 0.239x - 0.013	0.999	0.75 - 9.06	5.8	0.23	0.70	Fluka	43778
(−)-Linalyl acetate	y = 0.217x - 0.006	0.998	0.72 - 7.18	0.2	0.18	0.55	SAFC	w263605
Geraniol	y = 0.338x - 0.018	0.999	0.70 - 7.18	3.8	0.22	0.66	SAFC	w250716
Geranial	y = 0.372x - 0.022	0.994	0.85 - 5.59	11.7	0.28	0.85	SAFC	w230316
Bornyl acetate	y = 0.317x - 0.029	0.997	0.65 - 8.16	5.5	0.20	0.61	Fluka	45855
Thymol	y = 0.313x - 0.029	0.998	0.25 - 10.03	4.8	0.08	0.25	Sigma	T0501
Carvacrol	y = 1.069x - 0.118	0.997	0.65 - 10.35	1.3	0.21	0.65	SAFC	w224502
Neryl acetate	y = 0.242x - 0.007	0.999	0.73 - 7.31	3.8	0.18	0.55	SAFC	w277304
Geranyl acetate	y = 0.249x - 0.009	0.999	0.46 - 7.42	2.9	0.15	0.46	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(−)-(*E*)-β-Caryophyllene	y = 0.079x - 0.002	0.998	4.41 - 7.05	6.6	0.71	2.16	Sigma	22075
α-Humulene	y = 0.541x - 0.019	0.999	0.40 - 6.35	2.2	0.13	0.40	Aldrich	53675
(*E*)-α-Bisabolene	y = 0.111x - 0.043	0.999	1.74 - 6.97	3.4	0.31	0.94	Alfa Aesar	A18724
(−)-Caryophyllene oxide	y = 0.105x - 0.004	0.999	2.54 - 10.17	2.7	0.46	1.40	SAFC	w509647

[a] Response ratio vs. Concentration ratio, internal standard correction applied.
Each internal standard is reference compound for the analytes that follow.

Afterwards, 1 ml of 2.8 % (w/v) trichloroacetic acid and 1 ml of 1% (w/v) thiobarbituric acid were added to the test tube, which was boiled for 10 min to develop the pink coloured malonaldehyde–thiobarbituric acid (MDA-TBA_2) adduct. After cooling, its absorbance was measured at 520 nm.

Nitric oxide scavenging capacity

The nitric oxide (NO) scavenging activity of the samples was measured according to the method in the literature.[33] In this method, 50 µl of serially diluted samples were added to 50 µl of 10 mM sodium nitroprusside in phosphate-buffered saline (PBS). The reaction was carried out on a 96-well plate, incubating at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well, and the absorbance was read at 546 nm.

Reducing power

Each sample (300 µl) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $K_3[Fe(CN)_6]$ (2.5 ml, 1% w/v).[34] The mixture was incubated at 50 °C for 20 min. Next, 2.5 ml of 10% (w/v) trichloroacetic acid was added to the mixture,

which was then centrifuged for 10 min at 3000 rpm = 1000 g. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and with 0.5 ml, 0.1% (w/v) $FeCl_3$, and the absorbance was measured at 700 nm.

Thiobarbituric acid reactive substances assay (TBARS)

Egg yolk homogenate was used as lipid-rich media,[35] i.e. an aliquot of yolk material was made up to a concentration of 10% (w/v) in KCl (1.15%, w/v). The yolk was then homogenized for 30 s followed by ultrasonication for further 5 min. Next, 500 μl of the homogenate and 100 μl of the sample, dissolved in methanol, were added to a test tube and made up to 1 ml with distilled water, followed by the addition of 1.5 ml 20% (v/v) acetic acid (pH 3.5) and 1.5 ml 0.8% (w/v) TBA in 1.1% (w/v) sodium dodecyl sulfate (SDS). This mixture was stirred and heated at 95 °C for 60 min. After cooling at room temperature, 5 ml of butanol were added to each tube, stirred and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer.

Hyaluronidase inhibitory activity

Hyaluronidase inhibition was determined by measuring the amount of N-acetylglucosamine split from sodium hyaluronate.[36] Next, 50 μl of bovine hyaluronidase (7900 units/ml) were dissolved in 0.1 M acetate buffer (pH 3.6) and then mixed with 50 μl of designated concentrations of the essential oils and incubated for 20 min at 37 °C. Afterwards, 50 μl of 12.5 mM calcium chloride was added and incubated for 20 more minutes at 37 °C. This Ca^{2+} activated hyaluronidase was treated with 250 μl of 1.2 mg/ml sodium hyaluronate and incubated for 40 min at 37 °C. After incubation, 50 μl of 0.4 M sodium hydroxide and 100 μl of 0.2 M sodium borate were added to the reaction mixture and then incubated in boiling water for 3 min. After cooling to room temperature, 1.5 ml of PDMAB (*p*-dimethyl amino benzaldehyde) solution (4 g of PDMAB dissolved in 50 ml of 10 N HCl and 350 ml of glacial acetic acid) were added to the reaction mixture. The absorbance was measured at 585 nm in a UV spectrophotometer.

Statistical analysis

All data were recorded as the mean ± standard deviation of triplicate determinations. Each error value is magnified using the corresponding error propagation rules for arithmetic operations. Data quality was analysed by ANOVA,[37] and means were separated using Tukey's HSD considering significant differences for $P < 0.05$. Statistical analyses were conducted using SPSS Inc. (Chicago, IL, USA).

Results and discussion

The EOs were obtained by steam distillation in yields ranging from 0.2 to 1.5% (w/w). The chemical composition of the selected EOs is shown in Table 2A for the *Lavandula* group and Table 2B for the *Thymus* group. The listing order corresponds to the elution order in a non-polar SLB5-ms column.

The main components present in the studied *L. angustifolia* oil were linalool, α-terpineol, linalyl acetate and (*E*)-β-caryophyllene. Similarly, samples of plant material from Italy,[22,38] Greece,[2] Bosnia-Herzegovina,[24] Pakistan,[39] France[40] and Spain[41] show a high concentration of linalool and linalyl acetate. Just one of the studies mentioned above found 0.23% of thymol in *L. angustifolia* EO[39] (Table 2A). Other species of the *Lavandula* genus also show small amounts of thymol among its components such as *L. coronopofolia*.[42]

In the case of *L. latifolia* oil, the main components were β-pinene, 1,8-cineole, linalool, camphor and (*E*)-α-bisabolene. Other samples from Spain were found in the literature, whereas 1,8-cineole, linalool and camphor were common for all the cases, β-pinene was found among the main components only in the case of Valencia[5] and Castilla La Mancha.[41] The high concentrations of linalool and (*E*)-α-bisabolene found in our sample could be a peculiarity of the growing zone. Comparatively, our sample is very similar to the ones from Zaragoza[43] and the average of Spain.[44]

Lavandula hybrida samples show some differences between the two cultivars, 1,8-cineole, linalool, camphor, borneol, terpinen-4-ol and linalyl acetate being the main components of *L. hybrida* cv. Grosso oil. Moreover, the most abundant compounds present in *L. hybrida* cv. Super oil were (*Z*)-β-ocimene, 1,8-cineole, (*E*)-β-ocimene, linalool, camphor and linalyl acetate. Characteristic compounds of *L. hybrida* in the majority of samples are 1,8-cineole, linalool, camphor and linalyl acetate. Reports from Spain (Castilla La Mancha)[41] show a similar concentration of borneol to the studied sample, as well as the French report[45] which also shows terpinen-4-ol among the main components. The report from Turkey[46] shows ocimene like the studied samples, but the reports from Italy[38] and Greece[47] show different main compounds from the samples studied.

The main components of the *Lavandula* group were oxygenated monoterpenes, mainly alcohol (linalool), ester (linalyl acetate) and ether (1,8-cineole). *Lavandula angustifolia* showed the best yield in linalool production whereas both cultivars of *L. hybrida* showed the highest yields for linalyl acetate and terpinen-4-ol. The lowest yield of linalyl acetate and the highest of camphor and 1,8-cineole among the *Lavandula* group was found in *L. latifolia*. The high content of camphor found in *L. latifolia* and *L. hybrida* seems related to the content of sesquiterpenes of the caryophyllene type when compared with the low content of camphor shown in *L. angustifolia*, i.e. a high concentration of (*E*)-β-caryophyllene shows relation to low concentrations of camphor.[41,48]

Thymus zygis chem. thymol EO is the most studied case in the literature. In the studied sample, the main components were myrcene, α-terpinene, p-cymene, γ-terpinene, linalool, thymol and carvacrol. p-Cymene was found in all reported cases. Thymol and γ-terpinene were found among the main components in all reported cases except one from Portugal[14] and another from Almería (Spain).[11] Portuguese reports[15,16] are the only ones showing similar concentrations to the studied sample regarding myrcene and α-terpinene. The report from Jaén (Spain)[13] has a similar concentration of thymol. The studied sample is very similar to the ones from Jaén (Spain),[13] Córdoba (Spain),[49] Mirandela (Portugal)[16] and central Portugal,[15] and similar to the ones from the north of Portugal.[12,50]

In the case of the *T. hyemalis* chem. cineole EO, the main compounds were α-pinene, camphene, β-pinene, p-cymene, 1,8-cineole, linalool, camphor, borneol, terpinen-4-ol, α-terpineol and geraniol. p-Cymene and borneol are present in all studied literature, whereas β-pinene, terpinen-4-ol, α-terpineol and geraniol are just present among the main components in the studied sample. Just Spanish experimental crops samples[9] and the studied sample show linalool as a main component of the EO. Regarding Spanish samples, the one from Almería[8] is the most similar to

Table 2A. GC/MS determination and quantification of *Lavandula* sp. essential oils (EOs)

LRI (Lit.)	LRI (Exp.)	Analyte	Qualifying and quantitation ions[a] (m/z)	L. angustifolia Concentration (mM ± SD)	L. angustifolia Area (% ± SD)	L. latifolia Concentration (mM ± SD)	L. latifolia Area (% ± SD)	L. hybrida cv. Grosso Concentration (mM ± SD)	L. hybrida cv. Grosso Area (% ± SD)	L. hybrida cv. Super Concentration (mM ± SD)	L. hybrida cv. Super Area (% ± SD)	Identification methods
852	874	n-Hexanol	43, 56, 69, 82				0.1 ± 0.0		0.2 ± 0.0			LRI, MS
922	929	Tricyclene	79, 93, 121, 136		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0			LRI, MS
930	939	α-Pinene	39, 91, **93**, 77	3.2 ± 0.4	0.1 ± 0.0	96.7 ± 5.4	1.7 ± 0.0	14.5 ± 0.7	0.3 ± 0.0	31.9 ± 2.7	0.6 ± 0.0	LRI, MS, std
943	957	Camphene	67, 79, **93**, 121	17.4 ± 2.0	0.3 ± 0.0	38.8 ± 1.4	0.5 ± 0.0	12.7 ± 0.5	0.2 ± 0.0	20.9 ± 1.6	0.4 ± 0.0	LRI, MS, std
964	978	Sabinene	41, 77, 91, **93**			33.3 ± 1.7	0.6 ± 0.0				0.1 ± 0.0	LRI, MS, std
972	985	β-Pinene	41, 69, 79, **93**	5.0 ± 0.1	0.2 ± 0.0	120.5 ± 6.3	2.1 ± 0.0	12.0 ± 0.3	0.5 ± 0.0	27.3 ± 1.4	0.4 ± 0.0	LRI, MS, std
963	989	3-Octanone	**43**, 57, 71, 99	74.1 ± 3.1	0.5 ± 0.0					41.2 ± 2.0	0.3 ± 0.0	LRI, MS, std
981	992	Myrcene	39, **41**, 69, 93	92.4 ± 2.7	1.1 ± 0.0	61.9 ± 2.4	0.5 ± 0.0	56.3 ± 3.1	0.5 ± 0.0	77.5 ± 7.7	1.1 ± 0.0	LRI, MS, std
985	1002	3-Octanol	41, 59, 83, 101		0.1 ± 0.0						0.1 ± 0.0	LRI, MS
1008	1011	Hexyl acetate	**43**, 56, 84, 61	12.5 ± 0.1	0.2 ± 0.0			14.2 ± 0.8	0.1 ± 0.0	44.1 ± 1.4	0.5 ± 0.0	LRI, MS, std
1005	1014	3-Carene	77, 79, 91, **93**		0.1 ± 0.0						0.1 ± 0.0	LRI, MS
1011	1022	p-Cymene	77, 91, **119**, 134	4.0 ± 0.5	0.2 ± 0.0	6.9 ± 0.2	0.1 ± 0.0	6.0 ± 0.1	0.1 ± 0.0		0.1 ± 0.0	LRI, MS, std
1025	1026	Limonene	67, **68**, 79, 93	11.8 ± 1.7	0.5 ± 0.0	66.5 ± 7.7	1.1 ± 0.0	38.1 ± 1.0	0.6 ± 0.0	72.0 ± 1.7	1.0 ± 0.0	LRI, MS, std
1023	1030	1,8-Cineole	**43**, 67, 81, 93		0.0	1369.9 ± 25.5	29.7 ± 0.2	167.3 ± 5.6	3.6 ± 0.0	455.6 ± 22.9	7.9 ± 0.2	LRI, MS, std
1024	1034	(*Z*)-β-Ocimene	41, 79, 91, **93**	105.5 ± 2.7	0.9 ± 0.0			52.3 ± 2.8	0.6 ± 0.0	220.0 ± 6.7	2.5 ± 0.0	LRI, MS, std
1034	1045	(*E*)-β-Ocimene	41, 79, 93, 121		0.9 ± 0.0		0.1 ± 0.0		0.3 ± 0.0		2.6 ± 0.0	LRI, MS
1047	1050	γ-Terpinene	77, 91, **93**, 136			16.3 ± 0.9	0.2 ± 0.0	11.1 ± 0.1	0.2 ± 0.0	12.2 ± 0.2	0.1 ± 0.0	LRI, MS, std
1054	1055	*trans*-Sabinene hydrate	77, 91, **93**, 121		tr	13.7 ± 0.8	0.2 ± 0.0	8.5 ± 0.2	0.3 ± 0.0		0.1 ± 0.0	LRI, MS, std
1072	1070	*cis*-Linalool oxide (furanoid)	43, 59, 68, 111		0.4 ± 0.0							LRI, MS
1078	1083	Terpinolene	93, 105, 121, 136		0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.0		0.4 ± 0.0	LRI, MS
1087	1088	*trans*-Linalool oxide (furanoid)	43, 59, 68, 111		0.3 ± 0.0							LRI, MS
1103	1108	Linalool	41, 55, 69, **93**	5788.9 ± 69.0	55.6 ± 0.2	2421.1 ± 19.4	41.5 ± 0.1	2368.3 ± 41.7	41.7 ± 0.2	3205.1 ± 0.7	37.3 ± 0.1	LRI, MS, std
1097	1111	1-Octen-3-yl acetate	43, **54**, 67, 81	124.9 ± 0.2	0.4 ± 0.0							LRI, MS, std
1130	1133	allo-Ocimene	91, 105, 121, 136								0.2 ± 0.0	LRI, MS
1132	1152	Hexyl isobutyrate	43, 56, 71, 89				0.1 ± 0.0		0.1 ± 0.0		0.2 ± 0.0	LRI, MS
1142	1159	Camphor	41, 81, **95**, 108	91.6 ± 4.4	0.5 ± 0.0	881.2 ± 5.6	11.6 ± 0.2	683.1 ± 10.2	6.2 ± 0.1	385.9 ± 9.7	5.9 ± 0.0	LRI, MS, std
1170	1172	Lavandulol	41, 69, 111, 123		0.1 ± 0.0				0.7 ± 0.0		0.1 ± 0.0	LRI, MS
1174	1186	Borneol	41, 93, **95**, 121	181.8 ± 12.5	1.7 ± 0.0	74.0 ± 0.7	0.8 ± 0.0	396.8 ± 8.4	2.7 ± 0.0	115.3 ± 2.7	1.7 ± 0.0	LRI, MS, std
1175	1189	Terpinen-4-ol	71, 77, 91, **93**	27.6 ± 0.1	0.1 ± 0.0	23.9 ± 2.8	0.3 ± 0.0	359.7 ± 12.7	5.3 ± 0.0	60.0 ± 5.1	1.6 ± 0.0	LRI, MS, std
1176	1193	Hexyl butyrate	**43**, 56, 71, 89		0.2 ± 0.0	11.9 ± 0.4	0.1 ± 0.0	27.6 ± 1.5	0.5 ± 0.0	49.0 ± 1.2	0.9 ± 0.0	LRI, MS, std
1192	1206	α-Terpineol	59, 68, 79, **93**	194.0 ± 2.7	2.7 ± 0.0	70.1 ± 1.1	1.0 ± 0.0	64.1 ± 0.9	1.0 ± 0.0	80.6 ± 2.1	1.4 ± 0.0	LRI, MS, std
1232	1234	Hexyl 2-methylbutyrate	41, 57, 85, 103				0.1 ± 0.0				0.2 ± 0.0	LRI, MS

(Continues)

Table 2A. Continued

LRI (Lit.)	LRI (Exp.)	Analyte	Qualifying and quantitation ions[a] (m/z)	L. angustifolia Concentration (mM ± SD)	L. angustifolia Area (% ± SD)	L. latifolia Concentration (mM ± SD)	L. latifolia Area (% ± SD)	L. hybrida cv. Grosso Concentration (mM ± SD)	L. hybrida cv. Grosso Area (% ± SD)	L. hybrida cv. Super Concentration (mM ± SD)	L. hybrida cv. Super Area (% ± SD)	Identification methods
1235	1239	Hexyl valerate	43, 56, 85, 103				0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1232	1249	Geraniol	41, 69, 93, 121						0.5 ± 0.0		0.8 ± 0.1	LRI, MS
1236	1255	Linalyl acetate	41, 69, **93**, 121	1239.0 ± 2.9	22.8 ± 0.1	6.4 ± 0.1	0.1 ± 0.0	1569.9 ± 30.3	25.8 ± 0.1	1501.2 ± 22.2	25.7 ± 0.1	LRI, MS, std
1266	1284	Thymol	91, 115, **135**, 150		tr							LRI, MS, std
1272	1309	Lavandulyl acetate	43, 69, 93, 121		0.9 ± 0.0				1.9 ± 0.0		1.0 ± 0.0	LRI, MS
1342	1362	Neryl acetate	41, 69, 79, **93**	45.9 ± 0.1	0.6 ± 0.0			14.3 ± 0.1	0.3 ± 0.0	20.4 ± 0.0	0.4 ± 0.0	LRI, MS, std
1360	1380	Geranyl acetate	41, **69**, 79, 93	53.5 ± 0.1	1.1 ± 0.0			25.0 ± 0.7	0.5 ± 0.0	40.0 ± 0.5	0.7 ± 0.1	LRI, MS, std
1386	1387	β-Bourbonene	81, 123, 161, 189				0.1 ± 0.0					LRI, MS
1405	1405	α-Cedrene	93, 105, 119, 204				0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1412	1414	α-Gurjunene	105, 119, 189, 204				0.0 ± 0.0					LRI, MS
1421	1430	(*E*)-β-Caryophyllene	41, 69, 79, **93**	68.2 ± 2.4	3.3 ± 0.0	274.9 ± 3.7	1.5 ± 0.0	99.0 ± 1.5	1.2 ± 0.0	81.3 ± 0.2	1.3 ± 0.0	LRI, MS, std
1427	1441	α-Bergamotene	41, 69, 93, 119		0.7 ± 0.0		0.2 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1448	1461	β-Farnesene	41, 69, 93, 133		1.4 ± 0.1		0.3 ± 0.0		1.2 ± 0.0		0.6 ± 0.0	LRI, MS
1454	1469	α-Humulene	41, 67, 80, **93**			4.9 ± 0.0	0.1 ± 0.0	5.2 ± 0.2	0.1 ± 0.0	3.6 ± 0.0	0.1 ± 0.0	LRI, MS, std
1471	1489	γ-Muurolene	105, 119, 161, 204						0.5 ± 0.0		0.3 ± 0.0	LRI, MS
1490	1490	Germacrene D	105, 119, 161, 204		0.1 ± 0.0		0.7 ± 0.0					LRI, MS
1494	1504	(*Z*)-α-Bisabolene	41, 79, 93, 161				0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1500	1507	α-Selinene	161, 175, 189, 204				0.1 ± 0.0		0.2 ± 0.0		0.1 ± 0.0	LRI, MS
1500	1514	β-Bisabolene	41, 69, 93, MS									
1516	1532	β-Sesquiphellandrene	41, 69, 93, 161						0.1 ± 0.0			LRI, MS
1534	1549	(*E*)-α-Bisabolene	41, 79, **93**, 119			385.4 ± 26.2	2.3 ± 0.1					LRI, MS, std
1581	1597	Caryophyllene oxide	41, 79, **91**, 105	21.5 ± 0.1	0.3 ± 0.0	9.5 ± 0.1	0.1 ± 0.0					LRI, MS, std
1646	1658	δ-Cadinol	105, 119, 161, 204				0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1665	1695	α-Bisabolol/ epi-α-bisabolol	41, 69, 93, 119						0.3 ± 0.0		0.3 ± 0.0	LRI, MS
		Monoterpene hydrocarbons			4.6		7.2		3.6		9.6	
		Oxygenated monoterpenes			86.7		85.2		90.5		84.6	
		Sesquiterpene hydrocarbons			5.4		5.7		3.7		2.6	
		Oxygenated sesquiterpenes			0.3		0.2		0.4		0.3	

LRI = Linear Retention Index obtained from the homologous series of n-alkanes (C7-C30). MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. tr = traces (<0,1%). SD = standard deviation. Results are expressed as mean ± standard deviation of three determinations.

[a] Quantitation ions are shown in bold.

LRI (Lit.) obtained from NIST08 database. LRI (Exp.) obtained experimentally.

Table 2B. GC/MS determination and quantification of *Thymus* sp. essential oils (EOs)

LRI (Lit.)	LRI (Exp.)	Analyte	Qualifying and quantitation ions[a] (m/z)	T. zygis high thymol Concentration (mM ± SD)	T. zygis high thymol Area (% ± SD)	T. hyemalis Concentration (mM ± SD)	T. hyemalis Area (% ± SD)	T. zygis high linalool Concentration (mM ± SD)	T. zygis high linalool Area (% ± SD)	Identification methods
928	929	α-Thujene	39, 77, **93**, 136	109.2 ± 6.0	1.7 ± 0.0	53.1 ± 2.8	0.9 ± 0.0	22.0 ± 1.3	0.3 ± 0.0	LRI, MS, std
930	938	α-Pinene	77, **93**, 105, 121	73.7 ± 5.2	1.3 ± 0.1	315.4 ± 16.1	7.7 ± 0.0	167.4 ± 10.1	3.9 ± 0.0	LRI, MS, std
943	955	Camphene	79, **93**, 107, 121	52.1 ± 3.3	0.6 ± 0.0	266.8 ± 11.1	4.3 ± 0.0	82.8 ± 3.6	1.0 ± 0.0	LRI, MS, std
964	976	Sabinene	41, 77, 91, 93				1.5 ± 0.0		0.9 ± 0.0	LRI, MS
972	982	β-Pinene	69, 77, **93**, 121	12.3 ± 0.8	0.3 ± 0.0	114.9 ± 3.4	2.8 ± 0.0	16.7 ± 0.7	0.4 ± 0.0	LRI, MS, std
963	985	3-Octanone	43, 57, 71, 99		0.1 ± 0.0				0.1 ± 0.0	LRI, MS
981	989	Myrcene	41, 69, 79, **93**	195.0 ± 10.6	2.3 ± 0.1	68.0 ± 2.0	0.9 ± 0.0	556.0 ± 9.8	6.5 ± 0.0	LRI, MS, std
985	1002	3-Octanol	41, 59, 83, 101				0.1 ± 0.0		0.1 ± 0.0	LRI, MS
997	1010	α-Phellandrene	77, **93**, 119, 136	23.1 ± 0.5	0.3 ± 0.0			33.3 ± 1.4	0.4 ± 0.0	LRI, MS, std
1005	1012	3-Carene	77, 79, 91, 93		0.1 ± 0.0					LRI, MS
1008	1019	α-Terpinene	77, **93**, 121, 136	106.4 ± 5.6	2.1 ± 0.1	33.6 ± 1.3	0.7 ± 0.0	410.4 ± 13.0	3.7 ± 0.0	LRI, MS, std
1011	1022	p-Cymene	91, 117, **119**, 134	834.5 ± 14.9	18.7 ± 0.5	252.5 ± 6.2	6.0 ± 0.1	120.1 ± 5.2	2.4 ± 0.0	LRI, MS, std
1025	1026	Limonene	**68**, 79, 93, 121	39.6 ± 0.9	0.5 ± 0.0	87.5 ± 6.6	1.2 ± 0.2	234.6 ± 11.6	3.0 ± 0.0	LRI, MS, std
1023	1030	1,8-Cineole	**43**, 81, 93, 108	12.1 ± 0.5	0.3 ± 0.0	1267.4 ± 34.0	35.8 ± 0.2	6.7 ± 0.3	0.2 ± 0.0	LRI, MS, std
1024	1034	(*Z*)-β-Ocimene	41, 79, **93**, 105	33.2 ± 0.9	0.2 ± 0.0	70.7 ± 0.6	1.1 ± 0.1	94.9 ± 4.6	0.7 ± 0.0	LRI, MS, std
1034	1045	(*E*)-β-Ocimene	41, 79, 93, 105		0.1 ± 0.0	40.4 ± 0.8	0.5 ± 0.0		0.1 ± 0.0	LRI, MS
1047	1051	γ-Terpinene	77, **93**, 121, 136	594.6 ± 4.3	11.4 ± 0.4	76.0 ± 2. 4	1.4 ± 0.0	394.2 ± 15.4	6.9 ± 0.1	LRI, MS, std
1054	1055	*trans*-Sabinene hydrate	77, **93**, 121, 136	29.8 ± 0.5	0.4 ± 0.0	76.8 ± 1.1	1.2 ± 0.0	128.6 ± 5.0	2.1 ± 0.0	LRI, MS, std
1072	1070	*cis*-Lilalool oxide (furanoid)	43, 59, 68, 111						0.1 ± 0.0	LRI, MS
1078	1083	Terpinolene	93, 105, 121, 136		0.1 ± 0.0		0.3 ± 0.0		1.4 ± 0.0	LRI, MS
1087	1088	*trans*-Lilalool oxide (furanoid)	43, 59, 68, 111						0.3 ± 0.0	LRI, MS
1103	1105	Linalool	41, 69, **93**, 121	207.9 ± 6.9	3.7 ± 0.0	297.2 ± 6.9	3.8 ± 0.0	2001.8 ± 44.9	44.4 ± 0.1	LRI, MS, std
1071	1110	β-Terpinene	77, 93, 121, 136		0.1 ± 0.0		0.8 ± 0.0		0.4 ± 0.0	LRI, MS
1132	1152	Hexyl isobutyrate	43, 56, 71, 89				0.5 ± 0.0		0.3 ± 0.0	LRI, MS
1142	1157	Camphor	81, **95**, 108, 152	27.2 ± 0.8	0.2 ± 0.0	185.0 ± 4.5	3.3 ± 0.0	20.6 ± 0.3	0.2 ± 0.0	LRI, MS, std
1170	1179	Lavandulol	41, 69, 111, 123				0.6 ± 0.0			LRI, MS
1174	1181	Borneol	41, **95**, 110, 121	66.8 ± 1.4	0.9 ± 0.0	484.8 ± 16.8	4.7 ± 0.2	140.3 ± 5.0	1.9 ± 0.0	LRI, MS, std
1175	1187	Terpinen-4-ol	71, **93**, 121, 136	26.6 ± 0.7	0.8 ± 0.0	99.0 ± 1.9	3.8 ± 0.0	381.5 ± 17.2	12.2 ± 0.0	LRI, MS, std
1192	1202	α-Terpineol	68, **93**, 121, 136	7.0 ± 0.4	0.1 ± 0.0	280.7 ± 22.9	3.7 ± 0.0	111.0 ± 6.8	1.6 ± 0.1	LRI, MS, std
1206	1210	*trans*-Dihydrocarvone	67, 95, 109, 152						0.2 ± 0.0	LRI, MS
1204	1214	Verbenone	91, **107**, 135, 150			50.8 ± 1.3	0.7 ± 0.0	12.3 ± 0.2	0.1 ± 0.0	LRI, MS, std
1206	1224	*trans*-Carveol	91, 105, 119, 134						0.1 ± 0.0	LRI, MS
1208	1230	Citronellol	41, **69**, 81, 95			32.0 ± 1.4	0.3 ± 0.0	8.0 ± 0.4	0.1 ± 0.0	LRI, MS, std
1231	1239	Methyl ether of carvacrol	77, **91**, 117, 134	13.6 ± 0.4	0.2 ± 0.0	42.8 ± 1.5	0.7 ± 0.0	6.3 ± 0.9	0.1 ± 0.0	LRI, MS, std
1232	1246	Geraniol	41, 69, 79, **93**			130.1 ± 2.7	3.0 ± 0.0	8.3 ± 0.1	0.1 ± 0.0	LRI, MS, std
1236	1256	Linalyl acetate	**41**, 69, 93, 121			15.3 ± 0.8	0.2 ± 0.0	44.1 ± 1.1	0.7 ± 0.0	LRI, MS, std
1245	1271	Geranial	41, **69**, 77, 91			64.7 ± 2.7	0.8 ± 0.0		tr	LRI, MS, std

(Continues)

Table 2B. Continued

LRI (Lit.)	LRI (Exp.)	Analyte	Qualifying and quantitation ions[a] (m/z)	T. zygis high thymol Concentration (mM ± SD)	T. zygis high thymol Area (% ± SD)	T. hyemalis Concentration (mM ± SD)	T. hyemalis Area (% ± SD)	T. zygis high linalool Concentration (mM ± SD)	T. zygis high linalool Area (% ± SD)	Identification methods
1266	1283	Thymol	91, 115, **135**, 150	3148.8 ± 10.5	48.2 ± 0.9	13.8 ± 0.2	0.1 ± 0.0	16.8 ± 0.9	0.1 ± 0.0	LRI, MS, std
1272	1288	Lavandulyl acetate	43, 69, 93, 121				0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1275	1309	Carvacrol	77, 91, **135**, 150	136.2 ± 5.6	3.2 ± 0.2	72.3 ± 3.0	1.9 ± 0.1		tr	LRI, MS, std
1285	1315	Bornyl acetate	43, **95**, 121, 136			36.1 ± 1.6	0.8 ± 0.0	8.1 ± 0.8	0.1 ± 0.0	LRI, MS, std
1350	1350	α-Cubebene	105, 119, 161, 204				0.2 ± 0.0			LRI, MS
1360	1379	Geranyl Acetate	41, 69, **93**, 121			53.4 ± 1.1	1.2 ± 0.0		tr	LRI, MS, std
1421	1427	(*E*)-β-Caryophyllene	41, 79, **93**, 133	37.2 ± 1.1	1.6 ± 0.0	17.8 ± 0.4	0.8 ± 0.0	23.2 ± 1.2	1.0 ± 0.0	LRI, MS, std
1445	1447	Aromadendrene	91, 133, 161, 204		0.1 ± 0.0					LRI, MS
1454	1465	α-Humulene	80, 93, 121, 147		0.1 ± 0.0		0.1 ± 0.0		tr	LRI, MS, std
1492	1496	Ledene	107, 135, 171, 204		0.1 ± 0.0		0.2 ± 0.0			LRI, MS
1514	1527	δ-Cadinene	134, 161, 189, 204		0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1579	1562	Sesquisabinene hydrate	69, 119, 161, 207				0.1 ± 0.0			LRI, MS
1581	1594	Caryophyllene oxide	41, 79, **91**, 105	15.0 ± 1.9	0.1 ± 0.0	22.8 ± 0.7	0.2 ± 0.0	10.9 ± 1.2	tr	LRI, MS, std
1624	1647	Z-α-Copaene-8-ol	119, 132, 145, 159				0.1 ± 0.0			LRI, MS
		Monoterpene hydrocarbons			39.8		30.1		32.0	
		Oxygenated monoterpenes			58.0		66.7		64.7	
		Sesquiterpene hydrocarbons			2.0		1.4		1.0	
		Oxygenated sesquiterpenes			0.1		0.4		0.0	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. tr = traces (<0,1%). Results are expressed as mean ± standard deviation of three determinations.

[a] Quantitation ions are shown in bold. LRI (Lit.) obtained from NIST08 database. LRI (Exp.) obtained experimentally.

Table 3. Chiral determination of *Lavandula* and *Thymus* sp. essential oils (EOs)

t_R (min)		Analyte (X)	*L. angustifolia*		*L. latifolia*		*L. hybrida cv.* Grosso		*L. hybrida cv.* Super		*T. zygis* high thymol		*T. hyemalis*		*T. zygis* high linalool	
			(+) - [X]	(−) - [X]	(+) - [X]	(−) - [X]	(+) - [X]	(−) - [X]	(+) - [X]	(−) - [X]	(+) – [X]	(−) – [X]	(+) – [X]	(−) – [X]	(+) – [X]	(−) – [X]
(+) - X	(−) - X		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
6.20	6.13	α-Thujene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	39	61	N/A	N/A	N/A	N/A
7.79	7.52	α-Pinene	95	5	95	5	95	5	95	5	95	5	81	19	82	18
8.47	8.24	Camphene	5	95	27	73	21	79	5	95	N/A	N/A	5	95	N/A	N/A
8.89	9.16	β-Pinene	95	5	65	35	95	5	95	5	N/A	N/A	47	53	N/A	N/A
10.36	9.39	α-Phellandrene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5	95	N/A	N/A	N/A	N/A
10.52	10.00	Limonene	95	5	95	5	95	5	95	5	95	5	95	5	95	5
14.28	14.51	*trans*-Sabinene hydrate	95	5	95	5	95	5	95	5	95	5	95	5	95	5
15.73	15.57	Linalool	4	96	5	95	5	95	4	96	5	95	5	95	1	99
16.72	16.46	Camphor	95	5	95	5	95	5	95	5	5	95	5	95	5	95
-	17.25	Linalyl acetate	5	95	5	95	5	95	5	95	N/A	N/A	N/A	N/A	N/A	N/A
18.02	18.18	Bornyl acetate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5	95	N/A	N/A
18.32	18.51	Terpinen-4-ol	95	5	95	5	95	5	95	5	95	5	62	38	73	27
20.10	19.76	α-Terpineol	95	5	76	24	87	13	63	37	N/A	N/A	62	38	95	5
20.15	19.58	Borneol	61	39	60	40	95	5	95	5	5	95	5	95	5	95
23.92	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	5	95	5	95	5	95	5	95
-	28.81	Caryophyllene oxide	5	95	5	95	N/A	N/A	5	95	N/A	N/A	5	95	N/A	N/A

N/A = Not assessed

the studied sample, and the ones from designed experimental crops[7,9] are also similar to the studied sample. The Turkish sample[10] is the most different sample from the ones reported here.

The main components present in *T. zygis* with a high proportion of linalool EO were α-pinene, myrcene, α-terpinene, p-cymene, limonene, γ-terpinene, *trans*-sabinene hydrate, linalool and terpinen-4-ol. Myrcene, γ-terpinene, linalool and terpinen-4-ol were present in most of the reported samples in the literature, establishing, thus, the main common components. A high similitude was found between the studied sample, the Spanish sample from Jaén[13] and the experimental crops sample,[9] a higher difference can be found in the Spanish sample from Almería[8] and to the central Portugal sample.[15]

The main components in the case of the *Thymus* group (Table 2b) were oxygenated monoterpenes, mainly alcohols (thymol, linalool, terpinen-4-ol) and ether (1,8-cineole), and monoterpene hydrocarbons, γ-terpinene, p-cymene, α-pinene and β-myrcene showing the highest concentrations.

Thymus zygis with a high proportion of thymol was found richer in thymol, carvacrol and their biosynthetic precursors: γ-terpinene and p-cymene.[51] *Thymus hyemalis* shows a high concentration of 1,8-cineole and remarkable concentration of some of the products obtained from *ad latere* geranyl pyrophosphate reactions not leading to 1,8-cineole.[52,53] Those reactions are: geranyl pyrophosphate → geraniol → α-terpineol → terpinen-4-ol and geranyl pyrophosphate → α-pinene ↔ β-pinene → camphene → camphor → borneol. *T. zygis* with a high proportion of linalool is rich in linalool and terpinen-4-ol, and it has an interesting concentration of γ-terpinene not further developed into p-cymene and thymol.

Regarding the chiral determination (Table 3), (R)-(−)-linalool is shown in all determinations, as reported from Turkish EOs.[23,54] The same case happens with (R)-(−)-linalyl acetate or (S)-(+)-terpinen-4-ol reported in samples from Germany.[21] However, in the studied sample, all commercially available chiral compounds were examined, finding that (S)-(−)-camphene had lower concentration in the samples of *L. latifolia* and *L. hybrida* cv. Grosso among *Lavandula* group. Interestingly, (R)-(+)-β-pinene and (1R,2S)-(+)-borneol are found in lower concentration in *L. latifolia* and *L. angustifolia*, respectively, *L. hybrida* being a good source of pure (1R,2S)-(+)-borneol as reported in French and Swiss samples.[55] High variation is found in (R)-(+)-α-terpineol throughout the *Lavandula* group.

In the case of *Thymus* samples (Table 3), just two of the determined enantiomers, i.e. (S)-(+)-terpinen-4-ol and (R)-(+)-α-terpineol, show some variation in concentration. According to the reported data from Israel,[55] (1S,2R)-(−)-borneol has a high purity in these *Thymus* species. Variations were found in some worldwide studies about enantiomers of *Thymus* sp. EOs,[56] in the studied samples (R)-(+)-α-pinene and (R)-(+)-limonene were found.

Thymus zygis with a high proportion of thymol sample obtained the best results for almost all the antioxidant assays (Table 4), just three assays showed better performance for other samples, i.e. both cultivars of *L. hybrida* showed the highest chelating power, and *L. angustifolia* provided the highest $OH^{\bullet}$ and $ROO^{\bullet}$ scavenging activity.

The order of the tested samples in the reducing power assay (Figure 1) is: *T. zygis* high thymol > *T. zygis* high linalool ≈ *T. hyemalis* > *L. hybrida* cv. Super > *L. hybrida* cv. Grosso ≈ *L. latifolia* ≈ *L. angustifolia*. Thymol may have the best reducing activity because it was determined as the main component of *T. zygis*

Table 4. Results of the antioxidant and the hyaluronidase inhibition assays for essential oils (EOs)

Sample	IC_{50} (mg/ml)				TEAC (μmol TE/g EO)			Inhibition degree (%)[a]
	Chelating power	Hydroxyl	Nitric oxide	TBARS	ABTS	DPPH	ORAC	Hyaluronidase
Lavandula angustifolia	0.44b ± 0.09	0.206c ± 0.003	11b ± 2	0.29c ± 0.02	103.0b ± 0.7	1.35b ± 0.02	1984a ± 106	11c ± 3
Lavandula latifolia	0.62a ± 0.04	0.27c ± 0.05	24a ± 4	1.50b ± 0.06	1.51d ± 0.01	0.42c ± 0.01	1272d,e ± 74	6c,d ± 2
Lavandula hybrida cv. Grosso	0.08d ± 0.01	0.30b,c ± 0.02	N/A	2.5a ± 0.3	1.13d ± 0.03	0.04d ± 0.01	1407c,d ± 90	0d ± 0
Lavandula hybrida cv. Super	0.07d ± 0.01	0.27c ± 0.04	19a ± 4	1.3b ± 0.2	1.62d ± 0.03	0.216c,d ± 0.003	1388c,d ± 60	0d ± 0
Thymus zygis high thymol	N/A	0.44b ± 0.08	1.3c ± 0.1	0.074c ± 0.002	1061a ± 10	27.7a ± 0.3	1636b ± 54	100a ± 0
Thymus hyemalis	0.239c ± 0.001	0.23c ± 0.01	1.6c ± 0.1	0.4c ± 0.1	22.9c ± 0.4	0.46c ± 0.02	1087e ± 55	32b ± 7
Thymus zygis high linalool	0.35b ± 0.01	0.85a ± 0.07	9.6b ± 0.1	0.08c ± 0.03	10.6d ± 0.9	N/A	1546b,c ± 76	22b ± 6
Standard[b]	0.061d ± 0.001	0.001d ± 0.15	0.06c ± 0.05	0.06c ± 0.01				

[a] at 0,781 μlEO/ml;
[b] Standards used: EDTA for chelating power, Mannitol for hydroxyl radical, Rutin for nitric oxide, BHT for TBARS; a, b, c, d, e different letters in the same antioxidant column mean statistically significant differences with $p < 0.05$. N/A = Not assessed.

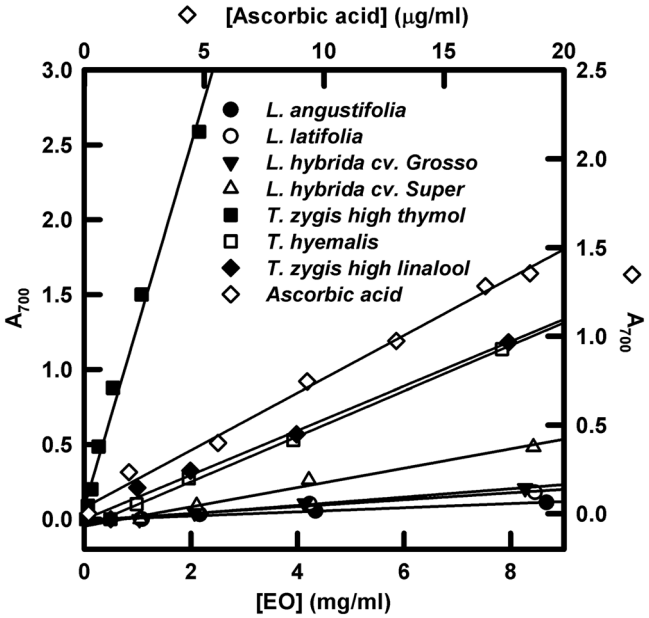

Figure 1. Reducing the power assay. Comparative for the assayed essential oils (EOs) (bottom and left axes), and ascorbic acid as standard (top and right axes)

high thymol. Positive results also agree with that reported for *T. hyemalis* from Turkey.[10]

The high performance of *L. hybrida* followed by *T. hyemalis* in chelating power (Table 4) can be explained by the high contribution of ester and ether groups to the general composition of the EOs. The case of *L. angustifolia*, showing high values in the hydroxyl and peroxyl radical scavenging assays, is explained by the high concentration of alcohol and ester groups, i.e. mainly linalool and linalyl acetate, respectively.

The results for the DPPH assay (Table 4), reveal that *L. angustifolia* has a better performance than *L. hybrida* cv. Super, in accordance with that reported for EOs from Italy.[38] The best performances showed by *T. zygis* high thymol and *T. hyemalis* in some antioxidant methods (Table 4), i.e. nitric oxide, ABTS and DPPH scavenging capacity, may be as a result of the concentrations of the common molecules thymol and carvacrol existing in those two samples. The phenolic molecule thymol is the main component of *T. zygis* high thymol, thus, we can attribute the antioxidant activity shown in the rest of the assays to the concentration of thymol, as already reported for TBARS in EOs from Portugal.[14] Nevertheless, in the case of TBARS (Table 4), *T. zygis* high linalool, not containing thymol or carvacrol, shows a low IC_{50} as well as *L. angustifolia*, showing a good performance of the pair: linalool-linalyl acetate, in this test.

The good performance obtained using *T. zygis* high thymol in the hyaluronidase inhibition assay (Table 4), mainly as a result of thymol concentration (Table 2B), agreed with the reported results for *Thymus* sp. EOs from Japan.[57] The relevant anti-hyaluronidase activity of EOs of *T. hyemalis* and *T. zygis* high linalool, as well as the weak inhibitory activity of *L. angustifolia* and *L. latifolia* EOs, could be related to their respective contents in α-pinene, camphene and α-terpineol (Tables 2A, 2B and 4).

Therefore, the EO of *T. zygis* with high proportion of thymol could be used as hyaluronidase inhibitor to prevent the hyaluronic acid fragmentation, which has dual effects, generation of oligosaccharides with angiogenic pro-inflammatory and immunostimulatory properties; and impairment in the reservoir capacity of ECM that holds oligoelement cations, growth factors, cytokines and several enzymes for signal transduction. Thus, the EO of *T. zygis* with a high proportion of thymol could aid to overcome the high number of diseases, related to the imbalance of the hyaluronic acid homeostasis.[25]

Conclusions

A sound and quantitative study of EOs of *Lavandula* sp. and *Thymus* sp. from Murcia country has been carried out. The main components of the *Lavandula* group were oxygenated monoterpenes, mainly alcohol (linalool), ester (linalyl acetate) and ether (1,8-cineole), whereas the main components in the case of *Thymus* group were oxygenated monoterpenes, mainly alcohols (thymol, linalool, terpinen-4-ol) and ether (1,8-cineole) and monoterpene hydrocarbons, with γ-terpinene, p-cymene, α-pinene and β-myrcene as the most abundant.

In *Lavandula* sp. EOs, there are high proportions of eight (+)-enantiomers, terpinen-4-ol, β-pinene, borneol and α-terpineol among them, and five (−)-enantiomers, linalool, linalyl acetate and camphene among them, those mentioned are especially relevant for their variability or purity as commented on in the Results and Discussion section.

In *Thymus* sp. EOs there are high proportions of five (+)-enantiomers, α-pinene, limonene, terpinen-4-ol and α-terpineol among them showing variations useful for species differentiation of the samples, and 10 (−)-enantiomers borneol among them, unveiling new sources of enantiomeric pure compounds.

Linalool-linalyl acetate combination is deduced to be effective. It is found in a high concentration in *Lavandula angustifolia* EO, and it showed good results when tested against hydroxyl radical, peroxyl radical and azo radicals like DPPH and $ABTS^{\bullet+}$.

Thymus zygis with a high proportion of thymol obtained the best results from the antioxidant assays. The phenolic molecule thymol is the main component of *Thymus zygis* high thymol, thus it is acceptable to attribute the antioxidant activity shown in the assays to the high concentration of thymol.

The anti-hyaluronidase activity of EOs is weak in *L. angustifolia* and *L. latifolia*, relevant in *T. hyemalis* and *T. zygis* with high proportion of linalool, and high in *T. zygis* with a high proportion of thymol.

The EOs of *Lavandula* sp. and *Thymus* sp., obtained from plants grown in Murcia country, have potential applications in the development of fragrances, flavours, cosmetics and drugs, they can be especially useful for the treatment of diseases related to oxidative stress, extracellular matrix breakdown and hyaluronic acid homeostasis.

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