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Use of ethyl lactate to extract bioactive compounds from *Cytisus scoparius*: Comparison of pressurized liquid extraction and medium scale ambient temperature systems

Marta Lores [a,*], Marta Pájaro [a], Marta Álvarez-Casas [a], Jorge Domínguez [b], Carmen García-Jares [a]

[a] Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Química. Universidade de Santiago de Compostela, Avda. das Ciencias s/n, Campus Vida, E-15782 Santiago de Compostela, Spain
[b] Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, E-36310 Vigo, Spain

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ABSTRACT

An important trend in the extraction of chemical compounds is the application of new environmentally friendly, food grade solvents. Ethyl lactate (ethyl 2-hydroxypropanoate), produced by fermentation of carbohydrates, is miscible with both hydrophilic and hydrophobic compounds being a potentially good solvent for bioactive compounds. Despite its relatively wide use as a general solvent, the utilization of ethyl lactate as an extraction solvent has only recently been considered. Here, we evaluate the possible use of ethyl lactate to extract phenolic compounds from wild plants belonging to *Cytisus scoparius*, and we compare the characteristics of the extracts obtained by Pressurized Solvent Extraction (the total phenolics content, the antioxidant activity and the concentration of the major polyphenols) with those of other extracts obtained with methanol. In order to explore the industrial production of the ethyl lactate *Cytisus* extract, we also evaluate medium scale ambient temperature setups. The whole plant and the different parts (flowers, branches, and seed pods) were evaluated separately as potential sources of polyphenols. All extracts were analyzed by LC–MS/MS for accurate identification of the major polyphenols. Similar phenolic profiles were obtained when using ethyl lactate or methanol. The main bioactives found in the *Cytisus* extract were the non-flavonoid phenolic compounds caffeic and protocatechuic acids and 3,4-dihydroxybenzaldehyde; the flavonoids rutin, kaempferol and quercetin; the flavones chrysin, orientin and apigenin; and the alkaloid lupanine. Regarding the comparison of the extraction systems, although the performance of the PLE was much better than that of the ambient-temperature columns, the energy consumption was also much higher. Ethyl lactate has resulted an efficient extraction solvent for polyphenols from *C. scoparius*, yielding extracts with high levels of plant phenolics and antioxidant activity. The antimicrobial activity of these extracts was also tested, showing antibacterial activity against Gram +ve bacteria. Qualitatively similar extracts were obtained either by using PLE or medium-scale-ambient-temperature systems, these last rendering larger volumes of extract with lower energy cost. Good results have been obtained with whole plant extracts; nevertheless, extracts enriched in a particular polyphenol can be obtained from different parts of the plant.

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1. Introduction

The solvents usually used to extract plant phenolics, e.g. ethanol, water or mixtures of these, are classified as generally recognized as safe (GRAS). However, one of the most important trends in the extraction of bioactive compounds is the search for and the application of new environmentally friendly, PLE food grade solvents. In this regard, the possible use of bioethanol and ethyl lactate as extraction solvents has been explored [1].

Ethyl lactate (ethyl 2-hydroxypropanoate) is a lactate ester. Lactate esters are used as food additives and in biochemicals, pharmaceuticals and cosmetic products. Some lactate esters have been used for many years in industrial applications, as solvents for nitro and ethyl cellulose, gums, oils, dyes, synthetic polymers and paints. As solvents, they can potentially be used as alternatives to glycol ethers and are non-ozone depleting and biodegradable [2]. On the basis of its thermophysical properties, ethyl lactate (EL) is described as a dense fluid of moderate viscosity, which makes it a very suitable candidate for application in different industrial fields [3].

Ethyl lactate is an environmentally friendly solvent and it meets eight of the 12 so-called principles of green chemistry [4]. It

* Corresponding author. Tel.: +34 881 814386; fax: +34 981 595012.
E-mail address: marta.lores@usc.es (M. Lores).

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is produced by fermentation of carbohydrate feedstock derived from the corn and soybean industries. The U.S. Food and Drug Administration [5] and the European Union [6] have approved its use in food products. It has a relatively high flashpoint and is colorless, environmentally benign, and completely biodegradable (releasing CO_2 and water) [7]. It is miscible with both hydrophilic and hydrophobic compounds and it is therefore a potentially good solvent for plant phenolics because of the wide range of polarities of the different families of polyphenols, ranging from benzoic and cinnamic acids to quercetins and other flavonols. In this regard, its possible use as a HPLC mobile-phase modifier has recently been applied [8].

Despite its relatively wide use as a general solvent, the utilization of ethyl lactate as an extraction solvent has only recently been considered. Very few applications consider the use of this solvent for Pressurized Solvent Extraction (PLE), and these are related to the extraction of carotenoids from different plant matrixes [7,9], the extraction of γ-linolenic acid from *Spirulina* [10], and the fractionation of edible oil compounds (squalene and tocopherol) [11,12].

Since its viability for the extraction of polyphenols has not yet been assessed, we evaluate the potential of ethyl lactate to extract plant phenolics from wild shrubs belonging to *Cytisus scoparius* Link. (family: Leguminosae). These brooms are widely distributed throughout the world, and they are considered a threat to other species in some countries. *Cytisus* spp. mainly grow in disturbed areas and their presence often favors the rapid spread of fire. The abandonment of large areas of land previously used for agriculture, the large number of forest fires that reduce tree presence (thus enabling the expansion of smaller species) and poorly designed forest campaigns all lead to the deforestation of large areas, which are quickly occupied by these shrubs [13]. The above factors favor the evaluation of these species as a source of bioactive polyphenols.

On the other hand, the infusion or decoction of leaves or flowers of plants of the genus *Cytisus* has been and is still being used in traditional medicine, mainly in Southern Europe [14–16] and Asia [17]. The plant is even used in ayurvedic medicine. The health benefits attributed to this ethnopharmacological agent are as diverse (anti-inflammatory, anti-haemorraghic, blood depurative, hypocholesterolemia, diuretic, anti-diabetic, cardiotonic, hepatoprotective, lithotriptic, diseases of the skin, hypnotic and sedative) as its chemical components, i.e. more than 70 phytoconstituents belonging to very different groups such as alkaloids, carotenoids, vitamins, sugars, fatty acids and plant phenolics [18].

Recent studies characterizing the polyphenols extracted from *Cytisus multiflorus* have reported high levels of different families of plant phenolics, particularly flavonols and flavones [16] and the individual phenolic composition of an ethanolic extract [19]. One study examined the carotenoid and flavonoid content of *C. scoparius* [20], but only considered branches; the proposed extraction and fractionation process is a multi-step procedure that produces acetone:water and ethanol:water extracts that do not contain the main polyphenols found in this study. Interestingly, the main goal of the aforementioned study [20] was to evaluate the extract for topical application to protect skin. None of the above studies used ethyl lactate as extraction solvent or PSE as an extraction technique.

In this manuscript, we evaluate the possible use of ethyl lactate to extract phenolic compounds from wild plants belonging to *C. scoparius*, and we compare the characteristics of the extracts obtained by Pressurized Solvent Extraction (the total phenolics content, the antioxidant activity and the concentration of the major polyphenols) with those of other extracts obtained with a more commonly used solvent, methanol. In order to explore the possibilities of an industrial production of the ethyl lactate *Cytisus* extract, we also evaluated another extraction system. The system is a medium scale ambient temperature setup originally designed to obtain polyphenols from white grape marc as an intermediate step to the final industrial scale, already developed in our research group and protected under patent applications [21,22].

2. Materials and methods

2.1. Chemicals

The material used as the dispersant phase was washed sea sand (200–300 μm, Scharlau, Barcelona, Spain). The extraction solvents used were ethyl lactate (> 98%, FCC, FG, by Sigma-Aldrich, Madrid, Spain) and methanol (LC–MS Chromasolv®, Fluka, Madrid, Spain). Formic acid (98–100%) (Merck, Darmstadt, Germany) was used in the chromatographic mobile phase. Ultrapure water was produced in the laboratory with a Milli-Q gradient system (Millipore, Bedford, MA, USA). The Folin&Ciocalteu phenol reagent was obtained from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate (Na_2CO_3, Panreac, Castellar del Vallès, Barcelona, Spain) and 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, St. Louis, USA) were used to determine the scavenging activity of the *Cytisus* extracts.

Pure standards of 3,4-dihidroxybenzaldehyde (34DHBA), caffeic acid, quercetin, isoquercetin (quercetin-3-glucoside) and rutin (quercetin-3-rutinoside) were all supplied by Fluka Chemie GmbH (Steinheim, Germany). Protocatechuic acid was obtained from WI ANALYTIC GmbH; orientin was obtained from Extrasynthese; apigenin was obtained from Carbosynth and chrysin from Alfa Aesar. All standards were purchased with a degree of purity greater than 95%, except isoquercetin (91.1%). Table 1 lists the names and CAS numbers of the studied compounds.

Individual standard stock solutions (1000–10,000 $\mu g\ mL^{-1}$) were prepared in methanol (MS/MS grade). Working solutions in water containing the target analytes (10–1000 $ng\ mL^{-1}$) were obtained by appropriate dilution. Stock and working solutions were stored in a freezer at -20 °C and were protected from light.

2.2. Samples

C. scoparius samples were collected locally (Santiago de Compostela, Spain) in spring. The samples were dried at room temperature, by hanging them for several days in a cool, dry place. The whole plants and the different parts (flowers, seeds, pods and branches) were evaluated separately as potential sources of polyphenols. Seeds were discounted for use due to their small size and difficult isolation. The pod samples include the seeds. All data were finally expressed in dry weight (dw).

2.3. Pressurized liquid extraction (PLE)

Extractions were performed with an Accelerated Solvent Extractor (ASE 150, Dionex, Co., Sunnyvale, CA, USA), equipped with 10-mL stainless steel cells and 60-mL collection vials. A cellulose filter (Dionex) was placed at each end of the PLE cell. Samples (0.5 g), previously ground in a mortar with the selected dispersant in a ratio 1:2, were placed in the 10 mL extraction cell, to which 1 g of clean sand (200–300 μm grain size, Sigma-Aldrich) had already been added. Any space in the cells was also filled with sand. The cell was tightly closed and placed in the PLE system. Extractions were performed without preheating the cell. The extraction pressure was set to 1500 psi, the flush volume was 60%, and the purge time was set to 100 s. The number of extraction cycles (2 cycles of 5 min each, in static mode) and the need for a cleaning step (discharged) were assessed in preliminary experiments. The extraction temperature was 120 or 180 °C, depending on the experiment. Extraction solvents were hydro-organic

Table 1
Identities, retention times and MS/MS experimental parameters for the target compounds.

Peak number	Compound name	CAS number	Retention time (min)	Parent ion (s) (*m/z*)	Product ion (s) (*m/z*)	Collision energy (eV)	Tube lens (V)	Polarity
1	Lupanine[a]	550-90-3	2.24	249	98.1	28	100	+
					114.0	26	100	+
					136.0	26	100	+
2	Protocatechuic acid (3,4-dihydroxy-benzoic acid)[b]	99-50-3	3.33	152.976	108.06	26	66	–
					109.07	16	66	–
3	Protocatechualdehyde (3,4-dihidroxy-benzaldehyde)[b]	139-85-5	3.38	137.069	91.09	24	75	–
					92.13	25	75	–
					108.13	24	75	–
					136.11	21	75	–
4	Caffeic acid (3,4-dihydroxy-cinnamic acid)[b]	331-39-5	5.37	178.978	134.01	28	102	–
					135.03	19	102	–
5	Orientin[b]	60485-60-1	7.41	447.160	327.14	23	135	–
					327.14	23	135	–
					357.16	22	135	–
					357.16	22	135	–
6	Kaempferol-hexoside[a]	1108717-10-7	8.93	477.000	283.90	40	137	–
					285.00	29	137	–
7	Isoquercetin (quercetin-glucoside)[b]	482-35-9	9.21	465.076	256.90	41	119	+
					302.97	14	112	+
8	Rutin (quercetin-rutinoside)[b]	153-18-4	9.23	609.182	178.88	44	105	–
					270.92	56	105	–
					300.01	37	105	–
9	Quercetin[b]	117-39-5	9.24	303.098	153.05	33	90	+
					229.11	28	119	+
11	Kaempferol-acetylhexoside[a]		10.48	489.000	284.00	40	127	–
					285.00	28	104	–
10	Kaempferol[b]	520-18-3	13.24	285.091	185.13	28	104	–
					187.14	31	104	–
					211.13	31	104	–
					239.11	29	104	–
12	Apigenin[b]	520-36-5	13.56	269.098	117.12	37	97	–
					149.12	26	97	–
					151.06	26	97	–
					225.14	23	97	–
13	Chrysin[b]	480-40-0	15.46	253.129	63.20	34	89	–
					143.18	30	89	–
					145.16	31	89	–
					209.19	24	89	–

[a] Identification based on mass spectrum and on literature data.
[b] Identification based on the comparison of the retention time and mass spectrum with those from pure standards.

mixtures containing different proportions of methanol or ethyl lactate. The extracts obtained were made up to a final volume of 25 mL with the corresponding organic solvent and then passed through a 0.45 µm polyvinylidene fluoride (PVDF) filter (Simple-pure, USA).

2.4. Medium scale ambient temperature systems

To evaluate the possibility of obtaining *C. scoparius* extracts at ambient temperature and using a scaling-up approach, the following systems were tested: a 35 cm height × 1.5 cm external diameter

glass column loaded with 20 g of sample (hereafter referred to as ×40, because the sample load is 40 times the amount used in PLE); and a 20 cm height × 5 cm external diameter glass column loaded with 50 g of sample (hereafter referred to as ×100 because the sample load is 100 times the amount used in PLE). The volume of the extracts obtained with the ×40 column was 50 mL and with the ×100 column, 150 mL. In each system, the selected amount of sample, previously ground in a mortar with the selected dispersant at a ratio of 1:2, was loaded onto the column, and the selected elution solvent was added and subsequently extracted.

2.5. Determination of total polyphenols (TPC)

The total polyphenols content (TPC) of *Citysus* extracts was determined according to the Folin–Ciocalteu (FC) colorimetric method [23]. TP contents were quantified from a calibration curve prepared with gallic acid standard solutions at concentrations ranging from 3 to 20 mg L^{-1} (R^2=0.9982) and expressed as mg of gallic acid equivalents in the liquid extract (mg GAE L^{-1}). TP sample concentrations were expressed as mg gallic acid per g of dry weight of the *Cytisus* sample (mg gallic g^{-1} dw).

2.6. Identification of polyphenols by LC–MS/MS

For accurate identification of the major polyphenols, the extracts were analyzed by LC–MS/MS, in a Thermo Scientific system (San Jose, CA, USA) consisting of a Quantum Access triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source. A Thermo Scientific Hypersil Gold aQ column (1.9 µm, 100 mm × 2.1 mm) was used at a flow rate of 300 µL min^{-1} and at 30 °C with an Accela HPLC pump/autosampler. The injection volume was 10 µL. The mobile phase solvents were (A) 0.1% formic acid/water and (B) 0.1% formic acid/methanol. The mobile phase gradient program started with 5% B, changed to 20% B at 2 min, to 70% B at 15 min and finally to 100% B at 16 min with a 3 min hold step. The entire HPLC with a flow rate of 300 µL min^{-1} and 30 °C column temperature.

The mass spectrometer and the HESI source were operated as follows: high purity nitrogen (99.9%) was used as sheath gas (at 35 au [arbitrary units]) and auxiliary gas (at 10 au) without ion sweep cone gas; the ion source vaporizer temperature was set to 350 °C; the capillary temperature was 320 °C; and the spray voltage was 3000 V in positive polarity and 2800 V in negative polarity. The ion source discharge current was 4.0 µA for both negative and positive polarities. The peak width was 0.4 Da in Q1 and 0.7 Da in Q3, and the argon pressure in the collision cell (Q2) was set to 0.002 mbar. For LC–MS/MS analysis, all SRM transitions were acquired in only one segment with a cycle time of 0.5 s.

Column effluent was monitored by Selected Reaction Monitoring (SRM). Polyphenols were detected in the negative ion mode thus mainly producing $[M–H]^-$ pseudomolecular ions for all the polyphenolic compounds except quercetins and the alkaloid lupanine, which were detected in positive ion mode (Table 1). The target polyphenols were introduced into the mass spectrometer by flow-injection, and the collision energies of the SRM transitions were optimized for each (Table 1). An extract of *C. scoparius* was also directly infused by flow-injection, and three more compounds, namely lupanine, kaempferol-hexoside and kaempferol-acetilhexoside were identified, thus enabling optimization of the corresponding MS parameters. Due to the lack of available standards, these three compounds could not be quantified and their response is shown relative to the most abundant extract obtained (as a percentage). For the other compounds, the first mass transition was used for quantification, and the second and following (where available) were used

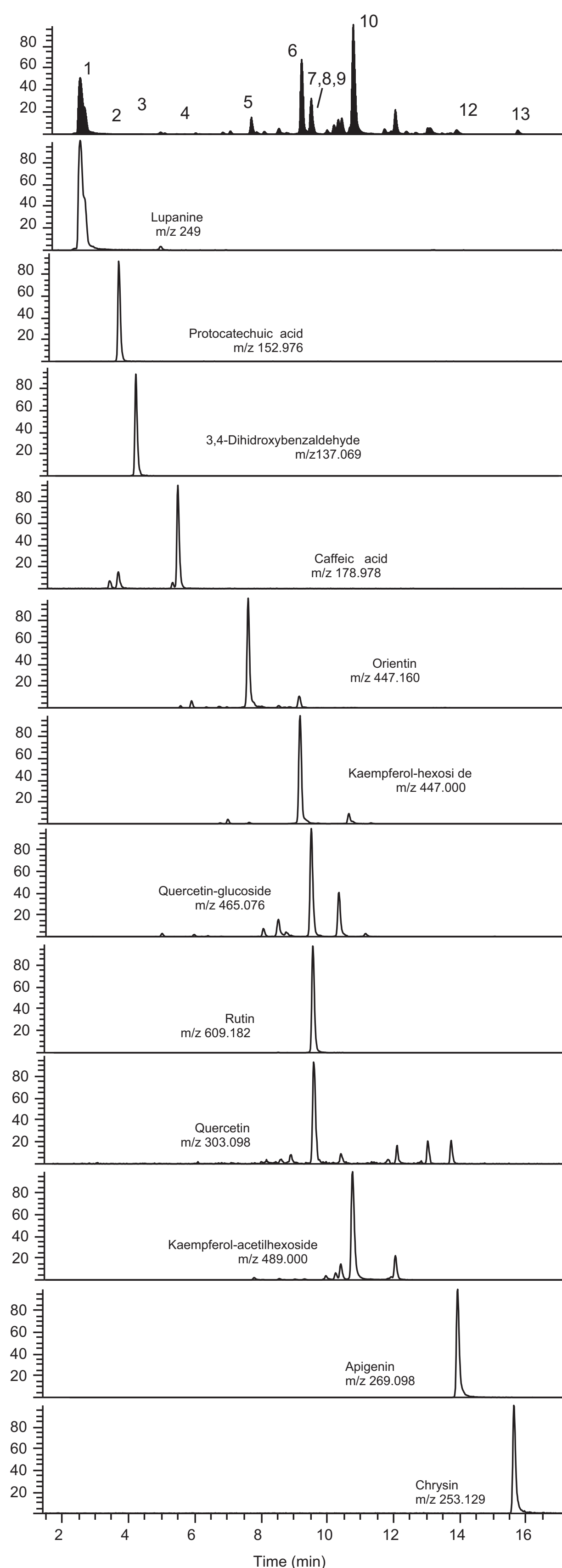

Fig. 1. LC–MS/MS reconstructed chromatogram and transition chromatograms for an ethyl lactate-derived extract of *C. scoparius* pods isolated on a *X*100 column. Only 12 of the 13 targets are shown because pod samples do not contain kaempferol (see concentrations in Table 2).

for identification/confirmation purposes; when only two transitions were available, the polyphenol identity was confirmed via these transitions and the retention time. Polyphenols were quantified using LC Quan software. External calibration was used for sample quantification. The calibration standards were obtained by serial dilution of a methanolic standard mixture in mobile phase. The linearity was assessed by use of standard solutions of between 10 and 1000 ng mL^{-1}.

The chromatographic profile (total ion chromatogram) of an ethyl lactate extract from *C. scoparius* and the extracted ion chromatograms for the target analytes are shown in Fig. 1. The identity of the 13 identified compounds and their retention times are listed in Table 1.

2.7. DPPH radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity was determined using a modified method of Brand-Williams et al. [24] against Trolox®. Briefly, 0.1 mM DPPH was dissolved in 100% methanol. An aliquot (0.1 mL) of each *Cytisus* extract was added to 3.9 mL of the methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The decrease in absorbance of the resulting solution after 30 min was measured at 515 nm. The antiradical activity (AA) was calculated using the following equation ($y=0.5223x+0.0276$; $R^2=0.999$), obtained from linear regression after plotting the absorbance at 515 nm (A_{515}) of known solutions of Trolox against concentration (0.08–1 mM). The DPPH radical scavenging activity of the extracts was expressed as mM Trolox g^{-1} of dry weight. The radical stock solution was prepared daily.

2.8. Evaluation of the antimicrobial activity

Staphylococcus aureus and *Bacillus* spp. (Gram+ve bacteria), *Escherichia coli* (Gram−ve bacteria) and *Candida albicans* (fungi) were used to evaluate the antibacterial activity of the *Cytisus* extracts. The culture media used were Luria Broth (LB) medium for bacterial strains and YPD medium for *C. albicans*. All media were supplemented with 2% agar-agar when required.

Liquid cultures were incubated in a shaker at 37 °C and 200 rpm, apart from *C. albicans*, which was grown at 28 °C. Solid medium used for bacteriostatic effect assays was supplemented with different final concentrations of the extract obtained in ethyl lactate:water (65:35). To test the bacteriostatic effect and the influence of the vehicle, appropriate quantities of the extract were transferred to different flasks containing 20 mL of melted nutrient agar, to obtain final concentrations ranging from 1% to 6% v/v. Control samples were prepared by transferring an equivalent amount of ethyl lactate to 20 mL of melted nutrient agar. The medium was then poured into quadruple sterile Petri dishes. Once solidified, plates were seeded with 50 µl of an overnight culture and incubated at 37 °C for 20–24 h. Cultures growing in nutrient agar plates were used as positive controls. Results are expressed as the minimum inhibitory concentrations (MICs).

3. Results and discussion

3.1. Comparison of ethyl lactate and methanol as extraction solvents

Preliminary tests were performed to assess the potential of ethyl lactate (EL) as an extraction solvent for obtaining polyphenols from *C. scoparius*, and EL extracts were compared with methanolic extracts obtained under the same conditions. Thus, PLE extractions were carried out with 50% MeOH or 50% EL in water, at a constant temperature of 120 °C. The TPC of both extracts was very similar: 31.73 ± 0.016 mg GAE g^{-1} dw for the hydromethanolic extract and 34.27 ± 0.008 mg GAE g^{-1} dw for the ethyl lactate in water elution mixture. The antioxidant activity was also very similar in both extracts: 3.77 ± 0.018 mM Trolox g^{-1} dw and 3.83 ± 0.004 mM Trolox g^{-1} dw. Hydroalcoholic mixtures, based on methanol or ethanol, have previously been validated as extraction solvents for polyphenols in studies characterizing other *Cytisus* species [16,19].

Considering the possibility of increasing the extraction temperature when using ethyl lactate as solvent (boiling point at 153 °C compared with 64.7 °C for methanol, both at atmospheric pressure), experiments with this solvent were also carried out at 180 °C, yielding 61.96 ± 0.032 mg GAE g^{-1} dw for the TP content and 6.86 ± 0.033 mM Trolox g^{-1} dw for the antiradical activity. Thus, the values of both parameters were higher when ethyl lactate was used as the solvent; these values were further increased by increasing the extraction temperature. Nevertheless, frequent extraction at high temperatures may damage the stability of the extraction cell joints (although the instrument is guaranteed to work at temperatures up to 200 °C). Therefore, in the following studies it was decided to use a temperature of compromise (120 °C), far enough from the upper limit of the equipment to prolong the life of consumables thereof, but high enough to ensure an efficient extraction that allows the adequate characterization of the extracts.

Table 1 lists the main compounds identified in both extracts by LC–MS/MS. The identity of all but three was confirmed with pure standards; the derivatives of kaempferol (hexoside and acetylhexoside) are proposed based on their mass spectra and literature data [16] as well as the identity of the alkaloid lupanine [18]. The presence of phenolic acids and 34DHBA has already reported for acetone:water and hydroethanolic extracts of *C. scoparius* branches [20], together with the flavonoids rutin, kaempferol and quercetin. Nevertheless, characteristic polyphenols of the genus *Cytisus* also include other flavonoids, i.e. the flavones chrysin, orientin and apigenin, and several of their derivatives [16,19]. Flavones were found in this study both in the methanolic and EL extracts, and they appeared in the extracts obtained from the whole plant and from all parts, although with different distributions, as will be shown below. Another bioactive compound, the alkaloid lupanine, was also found in the extracts, indicating that the experimental procedure for extracting polyphenols is also useful for obtaining substances belonging to other chemical families.

The similarity of the phenolic profile of the extracts obtained using mixtures of water with methanol or EL is of interest, because EL has potential industrial uses [4] in the food, cosmetics and even pharmaceutical [25] sectors. However, the methanolic extracts cannot be used, because the use of methanol is banned or limited in these industrial sectors. Moreover, none of the reported compositions of the hydroalcoholic extracts [16,19,20] included the above-mentioned flavones and the flavonols, phenolic acids and aldehydes together.

3.2. Comparison of whole plants and different parts as sources of bioactive polyphenols

Separate study of the polyphenolic contents of the different parts of the plant (branches, pods and flowers) and the whole plant was performed. The extracts were first obtained by PLE and then by using the medium scale ambient temperature systems under the conditions described in the materials and method section.

3.2.1. PLE

Two different compositions of the extraction solvent were initially evaluated: 65% of EL in water and 100% of EL, for comparison of the properties of a totally organic extract and a hydro-organic extract. The TPC and the antioxidant activity of the

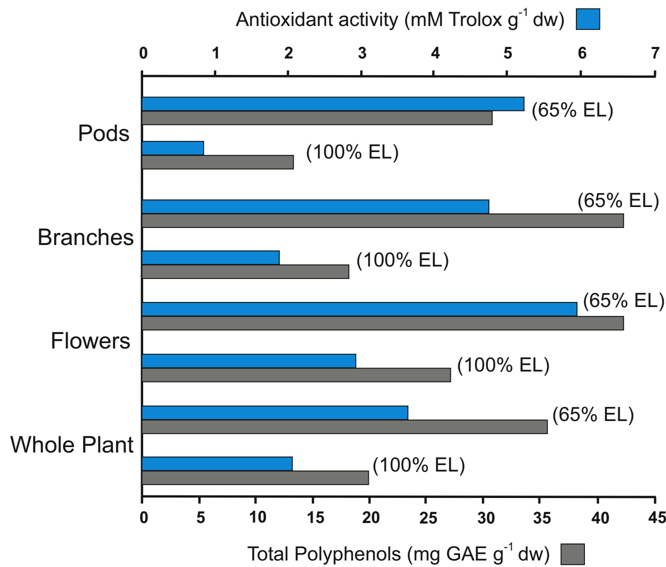

Fig. 2. Comparison of the antioxidant activities (upper axis) and total polyphenols content (lower axis) of PLE extracts from *C. scoparius*, obtained with different compositions of the extraction solvent (organic or hydro-organic).

extracts were much higher when a mixture of ethyl lactate and water was used as solvent (Fig. 2), independent of the raw material used to obtain the extract (part of the plant or the whole plant). The polarity of the EL is around 0.8 relative to that of water, 1.0 [26]. The molecular structure of EL offers diverse solvent properties that may cover a large number of solutes [27]. Thus, the EL:H_2O mixture will cover a wider range of polarities for polyphenol extraction, in particular increasing the potential extraction of the more polar compounds (e.g. benzoic and cinnamic acids). The viscosity of the solvent mixture is also lower than that of the 100% EL, thus improving the penetration and solubilization capacities of the extraction solvent and increasing the diffusion rates. A detailed study of the viscosity of this solvent has been carried out [3].

The LC–MS/MS analysis of the PLE extracts revealed different polyphenol contents as a function of the plant part considered (Table 2). Regarding the flavones, orientin is the most abundant and is characteristic of the pods; chrysin is the second most abundant flavone and is mainly found in the flowers along with apigenin, although this appears at a much lower concentration. The distribution of flavonols and their derivatives is even more specific to each plant part: rutin is the most abundant flavonol in PLE extracts and it is found in flowers. The flowers also contain appreciable amounts of quercetin and isoquercetin, but no

Table 2
Concentration of the individual polyphenols (ppb $\mu g\ L^{-1}$) and extraction efficiency (data expressed by dry weight) in the PLE extracts and in the extracts obtained by means of medium-scale-ambient-temperature systems.[a]

Extraction system	Raw material															
	Pods				Flowers				Branches				Whole plant			
	$\mu g\ L^{-1}$	SD	$\mu g\ g^{-1}$ dw	SD	$\mu g\ L^{-1}$	SD	$\mu g\ g^{-1}$ dw	SD	$\mu g\ L^{-1}$	SD	$\mu g\ g^{-1}$ dw	SD	$\mu g\ L^{-1}$	SD	$\mu g\ g^{-1}$ dw	SD
PLE																
3,4-DHBA	2199	343	110	17	374	13	18.7	0.6	313	43	15.7	2.2	388	16	19.4	0.8
Caffeic Acid	447	18	22.3	0.9	176	20	8.80	0.99	30	1	1.50	0.06	85	10	4.26	0.50
Protocatechuic	465	71	23.2	3.6	59	8	2.93	0.42	231	40	11.5	2.0	106	5	5.30	0.27
Chrysin	409	38	20.5	1.9	3434	154	172	8	556	34	27.8	1.7	2381	169	119	8
Apigenin	74	4	3.72	0.19	316	28	15.8	1.4	136	4	6.81	0.19	258	40	12.9	2.0
Orientin	4396	318	220	16	712	66	35.6	3.3	78	13	3.91	0.64	98	6	4.88	0.30
Kaempferol	6186	300	309	15	nd	–	nd	–	nd	–	nd	–	nd	–	nd	–
Q-3-glucoside	3.7	0.2	0.19	0.01	4838	508	242	25	nd	–	nd	–	1254	232	62.7	11.6
Rutin	nd	–	nd	–	11246	820	562	41	27	1	1.34	0.07	2200	417	110	21
Quercetin	445	43	22.2	2.2	4383	476	219	24	12	2	0.61	0.10	1400	281	70.0	14.0
× 100																
3,4-DHBA	5974	347	17.9	1.0	776	55	2.33	0.17	744	151	2.23	0.45	720	83	2.16	0.25
Caffeic Acid	1110	163	3.33	0.49	5658	497	17.0	1.5	855	29	2.57	0.09	2518	164	7.56	0.49
Protocatechuic	5393	339	16.2	1.0	565	35	1.69	0.10	93	12	0.28	0.04	158	17	0.47	0.05
Apigenin	1079	37	3.24	0.11	3782	144	11.3	0.4	2348	214	7.04	0.64	3160	250	9.48	0.75
Orientin	28,450	1987	86.5	6.0	9511	349	28.7	1.0	829	50	2.49	0.15	4742	369	14.2	1.1
Chrysin	10,462	172	31.4	0.5	15,291	375	45.9	1.1	6113	706	18.3	2.1	13,879	1557	41.6	4.7
Kaempferol	10,647	2146	31.9	6.4	nd	–	nd	–	nd	–	nd	–	nd	–	nd	–
Q-3-glucoside	nd	–	nd	–	81437	4526	244	14	118	15	0.35	0.05	26,660	2211	80.0	6.6
Rutin	nd	–	nd	–	49926	1596	150	5	459	62	1.38	0.19	26,238	912	78.7	2.7
Quercetin	2826	143	8.48	0.43	64332	3198	193	10	2424	127	7.27	0.38	19,899	2810	59.7	8.4
× 40																
3,4-DHBA	nm	–	nm	–	1352	100	3.38	0.25	1482	249	3.71	0.62	952	187	2.38	0.47
Caffeic Acid	nm	–	nm	–	1555	105	3.89	0.26	280	38	0.70	0.09	579	26	1.45	0.06
Protocatechuic	nm	–	nm	–	178	13	0.45	0.03	240	21	0.60	0.05	181	8	0.45	0.02
Apigenin	nm	–	nm	–	1241	149	3.10	0.37	107	21	0.27	0.05	440	87	1.10	0.22
Chrysin	nm	–	nm	–	6668	162	16.7	0.4	143	26	0.36	0.06	1271	125	3.18	0.31
Orientin	nm	–	nm	–	4857	346	12.1	0.9	500	59	1.25	0.15	1751	313	4.38	0.78
Kaempferol	nm	–	nm	–	nd	–	nd		nd	–	nd		nd	–	nd	
Q-3-glucoside	nm	–	nm	–	40,777	5636	102	14	225	39	0.56	0.10	9217	1269	23.0	3.2
Rutin	nm	–	nm	–	56,204	7700	141	19	94	17	0.24	0.04	3703	588	9.26	1.47
Quercetin	nm	–	nm	–	37,959	5689	94.9	14.2	280	17	0.70	0.04	4603	673	11.5	1.7

[a] nd = Not detected; nm = not measured; (–) SD missing values associated with not detected or not measured parameters.

kaempferol, which is only found in the pods. The pods do not contain any rutin or isoquercetin and only contain small amounts of quercetin. The whole plant shows a more homogeneous distribution of all flavonols except kaempferol, because the plants were collected before fruiting (with no pods in the samples). Branches are not a useful source of flavonols or their derivatives. Regarding the non-flavonoid polyphenols, 34DHBA is almost one order of magnitude more abundant in pods than the phenolic acids caffeic and protocatechuic; all of these compounds are more abundant in pods than in any other part of the plant. The relative response of the three compounds for which standards are not available is as follows: the quinolizidine alkaloid lupanine is found in branches and to a much lesser extent in flowers, and is almost nonexistent in pods. The flavonol derivatives kaempferol-hexoside and kaempfererol-acetylhexoside are found in proportions higher than 80% in branches, flowers and whole plants, while the pods do not contain these compounds. The latter two compounds have previously been described in *C. multiflorus* flowers [16].

All extracts obtained by PLE showed attractive characteristics from the point of view of their potential industrial applications. However, one of the disadvantages of this technique is the difficulty in scaling it up. Although fully automated instruments are available for carrying out unattended extraction of up to 24 samples simultaneously, the price of the equipment and the energetic consumption requirements make consideration of alternative extraction systems essential.

3.2.2. Medium scale ambient temperature systems

Given the aforementioned findings, the same approach (extraction with the hydro-organic mixture of 65% EL in water) was implemented in a series of experiments designed to develop a profitable scaled procedure to obtain EL-derived *Cytisus* extracts at ambient temperature to evaluate the potential for industrial exploitation of these extracts.

Two different extraction column systems with different sample loads were used: $\times 40$ and $\times 100$ (as described in Section 2). Table 2 summarizes the concentration of the individual polyphenols obtained with both setups, together with that obtained in the PLE experiments, differentiated by the part of the plant used as the raw starting material. The values indicate the amounts of the different polyphenolic compounds contained in the liquid extracts obtained by the different techniques tested, considering the potential use of the extracts directly in the liquid state. It should be emphasized that the columns ($\times 40$ and $\times 100$) yield extract volumes that are respectively twice and six times the volume obtained by PLE. Thus, for comparison of the extraction efficiencies, data were also expressed in dry weight (Table 2), as very different quantities of raw materials were processed in the different extraction systems.

Although the extraction performance of the PLE technique was much better than that of the ambient-temperature columns (Table 2, Fig. 3), the energy consumption was much higher. The possibility of obtaining large amounts of the extracts is limited by the difficulties in scaling up the PLE process, as already mentioned. Nevertheless, Fig. 3 clearly shows that the phenolic composition profiles obtained by means of the medium scale ambient temperature systems are equivalent to those achieved by PLE, irrespective of the part of the plant processed. We have combined all minority compounds to highlight this idea; however, the individual concentrations are shown in Table 2. Flowers and whole plants (Fig. 3) show very similar profiles; however, strict comparison reveals that less 34DHBA was extracted from branches by the $\times 100$ column, and less apigenin than expected was extracted with the $\times 40$ column. Because of an insufficient amount of sample, no $\times 40$ data is included in Fig. 3, which also shows the phenolic content of the pods; nevertheless, qualitatively very similar polyphenolic profiles were obtained by PLE and with the $\times 100$ column.

This same scaling up approach was previously used by our research group to evaluate the production of polyphenols from white grape marc. Adaptation from the $\times 100$ column to an industrial column is straightforward and the proposed process, protected under patent [21,22], is currently being used by a company producing natural ingredients for cosmetics. The processing of other materials such as *C. scoparius* (whole plants or parts) opens up new possibilities for obtaining extracts that are rich in certain polyphenols in particular or the full range of polyphenols associated with this plant species.

3.2.3. Properties of the main plant phenolics found in C. scoparius extracts

Table 2 and Fig. 3 summarize the main polyphenolic components of each plant part. Although all the polyphenols identified are associated with health benefits, some of the compounds are of particular interest.

Thus, flowers yield extracts rich in rutin, quercetin and isoquercetin. Rutin can interact with free radicals and various protein systems to exhibit antioxidant, anti-inflammatory, anti-allergy and

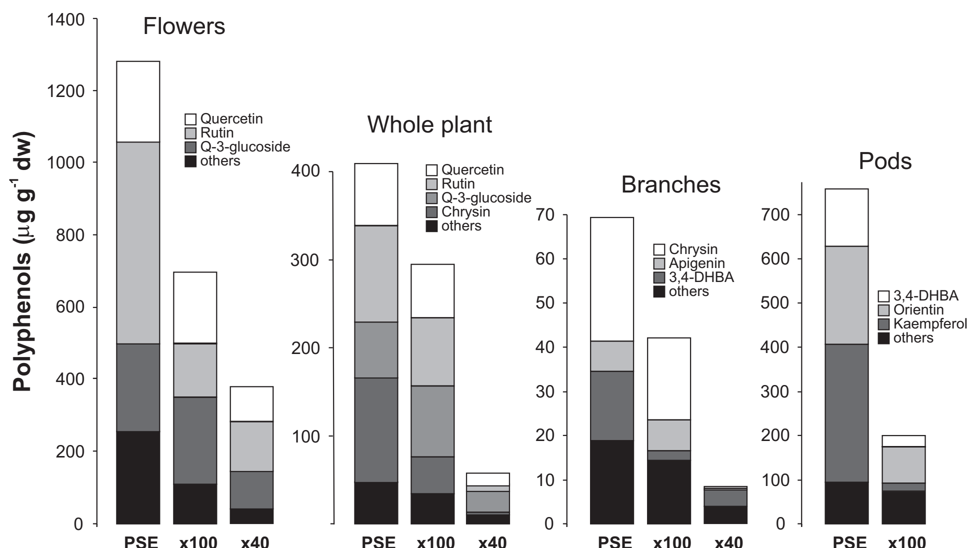

Fig. 3. Distribution of major polyphenols in *C.scoparius* extracts obtained from different starting materials (flowers, whole plant, branches and pods) in relation to the extraction technique used. The extraction solvent was 65% EL in water in all systems (note that *y*-axis are in different scale).

antitumor activity [28]. Isoquercetin has notable antifungal activity [29], and quercetin displays potentially anticancer properties [30].

The extracts from branches are rich in chrysin and apigenin. Both flavones were equally able to reduce locomotor activity in rats; however, while chrysin exhibited a clear anxiolytic effect, apigenin did not [31]. Nevertheless, besides having anti-inflammatory and antioxidant properties, apigenin has gained particular interest as a health promoting agent compared with other structurally-related flavonoids [32]. In addition to the anxiolytic effects of chrysin, this flavone has potent antioxidant and antimicrobial activities [33,34]. Lupanine is an alkaloid that mainly appears in branches too [18].

The pods contain important amounts of kaempferol and orientin. The health benefits associated with kaempferol have been also recently reviewed [35] highlighting its successful combination with conventional chemotherapeutic drugs. Interestingly, the historical use of kaempferol as a dyestuff material for textiles has recently been reported [36]. Orientin displays a wide variety of antiviral, antioxidant, antimicrobial and even radioprotection activity [37,38].

As already mentioned, the extracts from the whole plants contain all the target compounds except kaempferol, due to the absence of pods in the samples.

3.3. Antimicrobial activity

The antimicrobial activity of the *C. scoparius* extract was evaluated by determining the Minimal Inhibitory Concentrations (MICs), as described in Section 2. The extracts obtained by the $\times 100$ column in 65% EL in water were tested. The results show that the solvent (vehicle) plays an important role in the antibacterial behavior of the *C. scoparius* extracts, with a MIC of 3% by itself; this behavior differed depending on the microorganism evaluated. For Gram-positive species (*S. aureus* and *Bacillus* spp.) the MIC decreased from 3% to 2%, revealing that the polyphenols contained in the extract exerted a positive antibacterial action. Nevertheless, testing with a Gram-negative bacterium (*E. coli*) yielded an unexpected increase in the MIC from 3% to 4% when using the extract. This shows that the extract may confer some type of protective effect to this bacterial species. Finally, experiments with the fungus *C. albicans* produced the same MIC value (3%) for both the control and for the shrub extract.

These results are consistent with those published by Liu et al. [34] regarding the antimicrobial activity of some individual flavonoids contained in extracts of *Halostachys caspica* against Gram-positive bacteria of the same genera tested here; specifically, the polyphenols found in both extracts are chrysin, quercetin and isoquercetin. However, in relation to the antimicrobial activity against Gram-negative bacteria and fungi, the results are different from those obtained by the aforementioned authors. Regarding fungi, the species assessed are different in nature (spore and non-spore). It should also be noted that testing the antibacterial activity of the individual compounds isolated from the extract is not equivalent to testing a multicomponent extract, which may yield synergistic or antagonistic effects. Therefore, we believe that the present findings regarding the antibacterial activity of the hydro-organic extracts from *C. scoparius* are very interesting and promising. We are carrying out further research on this topic in collaboration with a microbiology research group at our university.

4. Conclusions

Ethyl lactate has resulted an efficient extraction solvent for polyphenols from *C. scoparius*. Analysis of the extracts obtained revealed high contents of plant phenolics and high levels of antioxidant activity. *Cytisus* therefore appears to be a suitable raw material for obtaining bioactive compounds. Good results have been obtained with whole plant extracts; nevertheless, extracts enriched in a particular polyphenol can be obtained from different parts of the plant. Furthermore, the determinations carried out showed the strong extraction capacity of ethyl lactate, which is comparable to or even better than that of other commonly used extraction solvents, with the additional advantage of being compatible with food, cosmetic and pharmaceutical uses. Qualitatively similar extracts were obtained either by using PLE or medium-scale-ambient-temperature systems, these last giving larger volumes of extract with lower energy cost. Thus, two options are proposed in this work: to get a more concentrated extract in low volume by PLE or to get a less concentrated extract ready to use in the liquid state; the latter obtained by a process definitely much easier to scale-up. Additionally, the antimicrobial activity of these extracts was tested, showing strong antibacterial activity against Gram +ve bacteria. To properly evaluate the activity against Gram −ve and fungi, additional experiments are currently being conducted.

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