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ORIGINAL ARTICLE

Cistus ladanifer as a source of chemicals: structural and chemical characterization

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Abstract

Different biomass fractions of *Cistus ladanifer* and solid residues from essential oil distilleries were structurally and chemically evaluated. The *C. ladanifer* biomass fractions showed chemical differences mainly related to extractives (e.g., 10.8% and 53.7% in stems and leaves) and lignin (e.g., 21.2% and 15.4% in stems and leaves). The distillery residues were characterized by 41.5% extractives and 19.3% lignin, and polysaccharide glucose 51.7% and xylose 24.9% of total monosaccharides. The polar extracts had a high content of phenolics and revealed high antioxidant activity (IC_{50} 3.2 μg/mL and 4.7 μg/mL in stems and cysts extracts).

The lignin structure showed a predominance of S units in the stem (H:G:S of 1:25:50) and a balanced proportion of H, G, and S units in leaves (H:G:S of 1:1.4:1).

The characteristics of *C. ladanifer* biomass allow several routes of valorization. The high extractive contents point out to the potential use as a source of phytochemicals by applying extraction procedures, while the remaining lignocellulosic material after extraction may be directed towards lignin and carbohydrates applications. The use of *C. ladanifer* biomass for an extractives-lignocellulosic-based biorefinery therefore represents a potential valorization that may contribute to additional revenue for the present essential oil distilleries.

Keywords Extractives · Antioxidant activity · Polysaccharides · Lignin · Biorefinery

Abbreviations

CL	*Cistus ladanifer*
CLR	Steam distillation residues
DPPH	1,1-Diphenyl-2-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
TEAC	Trolox equivalent antioxidant capacity
GAE	Gallic acid equivalent
QE	Quercetin equivalent
CE	Catechin equivalent
H	*p*-Hydroxyphenyl lignin monomeric unit
G	Guaiacyl lignin monomeric unit
S	Syringyl lignin monomeric unit
CZE	Capillary Zone Electrophoresis

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1 Introduction

Most genera of the Cistaceae family, including shrubs, semi-shrubs, and herbs, are distributed in the Mediterranean region where they occur especially in open areas and poor soils [1]. In particular, the genus *Cistus* of dicotyledonous perennial herbaceous plants is widespread and includes some species whose extracts have been used in folk medicine and as fragrances [2]. *Cistus ladanifer* (CL) is one of the main species producing labdane, a resin employed as a natural fixative and as a fragrance for composing amber and leathery notes, and also used as an incense [2, 3]. Other odoriferous materials may also be obtained from fresh leaves and branches: essential oil

by steam distillation, cistus concrete by nonpolar solvent extraction, and absolute by taking up concrete. These products are used in perfumery and come mainly from Spain [4–6].

The solid residues generated from the CL essential oil extraction process cannot be reused for gum extraction and vice-versa, but they constitute a lignocellulosic material that may be valorized in different ways, namely as a source of other extractives. In fact, the use of biomass within the biorefinery concept has seen a growing interest since it allows the production of a rich combination of products including biofuels, pulp and paper, biomaterials, and biochemicals. Residues such as bark and foliage are rich in phytochemicals that can be used as biopharmaceuticals, food additives and nutraceuticals, biopesticides, and cosmetics, although they still remain underexploited [7–9].

Several pharmacologically interesting compounds were already identified in *C. ladanifer* fractions, e.g., monoterpenes, sesquiterpenes and labdane-type diterpenes, flavonoids, phenolics, tannins, and carbonylic compounds [2]. The different extracts showed various biological properties: antioxidant [10–12], antibacterial [5, 13], antifungal [5, 14], cytotoxic [12], allelopathic [14, 15], antihypertensive [16], and hypoglycemic [17]. Only some works reported recently on the potential use of the *C. ladanifer* lignocellulosic material for obtaining carbohydrates and lignin-derived products and of *C. ladanifer* extractive fractions targeted as potential valuable chemicals within a biorefinery platform [18, 19].

It is acknowledged that the specific chemical and structural compositions of the different biomass fractions are important to design the various valorization pathways. This paper addresses this issue by dividing the *C. ladanifer* plant into the stem, branches, leaves, and cysts, and characterizing them, as well as the distillery residues obtained from two industries in Portugal. The chemical summative composition, the monomeric composition of polysaccharides and lignin, and the content, composition, and bioactivity of extractable compounds, as well as the anatomical structure of the stem, were studied. The objective is to characterize in detail the chemical and structural features of *C. ladanifer* and of its residues obtained from the industrial essential oil distillation in order to provide background data for their integration in biorefineries.

2 Materials and methods

2.1 Sampling

Cistus ladanifer plants aged 2 to 5 years were randomly selected and harvested from Quinta Essência (Portel, Portugal). These plants were fractionated into stem, branches, leaves, and cysts, and air-dried under well-ventilated conditions for 15 days. The *C. ladanifer* residues obtained after steam distillation (CLR) for extraction of essential oils were collected from two distillery units: Quinta Essência (Portel, Portugal) (CLR1) and SILVAPOR - Ambiente & Inovação, Lda., Quinta da Devesa (Idanha-a-Nova, Portugal) (CLR2). The plants used for the production of essential oils by these distilleries were between 2 and 4 years of age. Stems, branches, leaves, cysts, and CLR1 and CLR2 were ground with a knife mill with an output sieve of 6×6 mm^2. The chemical characterization was made on granulometric fraction 40–60 mesh (0.250–0.450 mm). The samples were stored in individual lots at room temperature.

2.2 Anatomical characterization

Stem samples from *C. ladanifer* aged 2 to 5 years were impregnated with DP1500 polyethylene glycol, and transverse sections of approximately 17-μm thick were prepared with a Leica SM 2400 microtome using Tesafilm 106/4106 adhesive for sample retrieval. The sections were stained with double staining of chrysodine/astra blue and mounted on Kaiser glycerin. After 24 h, the lamellas were submerged into xylol for 30 min to remove the Tesafilm, dehydrated in 96% and 100% ethanol, and mounted in Eukitt. Stem bark and wood samples were also macerated in a 1:1 solution of 30% H_2O_2 and CH_3COOH at 60 °C for 48 h and stained with safranine.

The microscopic observations were made with a Leica DMLA optical microscope, and the photomicrographs were taken with a Nikon FXA camera.

2.3 Chemical characterization

After milling to particles of < 1 mm, the ground material was sieved in a vibratory apparatus, and the 40–60 mesh fraction (0.250–0.425 mm) was recovered and used for the chemical analysis. The summative chemical composition of the samples was analyzed by sequential determination of extractives, lignin, and polysaccharide content. The inorganic content was quantified as ash.

Extractives were determined by successive Soxhlet extractions with pure solvents: dichloromethane, ethanol, and water as described before [19]. The extractives solubilized by each solvent were determined by the mass difference of the solid residue after drying at 105 °C and reported as a percent of the original sample [20]. Acid-insoluble (Klason) lignin and acid-soluble lignin were determined on the extracted samples by acid hydrolysis with 72% H_2SO_4: Klason lignin was determined as the mass of the solid residue after drying at 105 °C [21], and the acid-soluble lignin was determined in the filtrate by UV spectroscopy at 206 nm [22]. Total lignin was defined as the sum of the Klason and acid-soluble lignins. Measurements were reported as a percentage of the original sample. The remaining acid solution was kept for sugar analysis.

The polysaccharides were estimated by the content in neutral and acid monosaccharides (arabinose, xylose, galactose, mannose, glucose, and galacturonic acids) as well as acetic acid in the hydrolysate obtained from the lignin determination. High-performance ion chromatography with pulsed amperometric detection (HPIC-PAD) was used to quantify the monosaccharides in the hydrolysate of each sample, using a Dionex ICS-3000 system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA10 (250 × 4 mm) plus AminoTrap column. The separation was carried using a linear gradient of NaOH and CH_3COONa solutions as eluent at a flow rate of 1 mL/min (0 ± 20 min 18 mM NaOH; 20 ± 25 min 50 mM NaOH + 170 mM CH_3COONa); the column temperature was maintained at 30 °C. The content of acetic acid was also determined in the hydrolysate using a high-pressure ion-exclusion chromatography with a UV/visible detector (HIPCE-UV 210 nm) using a Thermo Finnigan Surveyor equipped with a Bio-Rad Aminex 87H column (300 × 7.8 mm). The separation was achieved using a 10 mM H_2SO_4 mobile phase at a flow of 0.6 mL/min. The column temperature was 30 °C.

The ash content was determined according to TAPPI standard [23], by incinerating 2.0 g of material at 525 °C overnight, and the residues were weighed and reported as a mass percentage of the original samples.

2.4 Composition of ethanol–water extracts

Extracts were prepared using approximately 1-g sample and ethanol/water (50/50, *v*/*v*), with a 1:10 (*m*/*v*) solid to liquid ratio for 60 min at 50 °C using an ultrasonic bath. After filtration, the solubilized extractives were determined by the mass difference of the solid residue after drying at 105 °C and reported as a percent of the original sample. The supernatant extract was used for the determination of the phenolic profile and quantitative analysis of total phenolics, flavonoids, and condensed tannins, and for the determination of the antioxidant activity. Each assay was performed at least three times, and at least three independent replicates were prepared for each standard and sample. The phenolic profile was obtained by capillary zone electrophoresis (CZE), according to the method described in [24]. Compounds were detected at 280 nm and analyzed by comparison of their UV spectra and migration times using authentic standards.

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method [25]. An aliquot (100 μL) of the extract was mixed with 4 mL of the Folin–Ciocalteu reagent and 4 mL of 7% Na_2CO_3 solution. The mixture was kept for 5 min at 50 °C, and, after cooling, the absorbance at 760 nm was measured. A calibration curve was built using gallic acid as a standard (0–150 μg/mL). The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

Total flavonoids content was determined using the aluminum chloride colorimetric method [26]. The calibration curve was prepared with catechin. An aliquot (1.0 mL) of the extract was mixed with 4.0 mL of deionized water and 0.3 mL of 5% $NaNO_2$ solution. After 5 min, 0.3 mL of 0% $AlCl_3{\cdot}6H_2O$ solution was added to the mixture. After 5 min, 2.0 mL of 1 M NaOH solution was added, and absorbance at 510 nm was measured. The total flavonoid content was calculated as milligrams of (+)-catechin equivalents (CE) per gram of the dry extract.

Tannins content was determined by the vanillin-H_2SO_4 method [27]. The calibration curve was prepared with catechin. An aliquot (1.0 mL) of the extract was incubated for 15 min in the presence of 2.5 ml of 1.0% (*m*/*v*) vanillin and of 2.5 ml of 25% (*v*/*v*) sulfuric acid, both diluted in absolute methanol. The blank solution was prepared without vanillin. The absorbances were measured at 500 nm, and results were expressed as milligrams of catechin equivalents (CE) per gram of the dry extract.

2.5 Antioxidant activity

Two methods were used to determine the antioxidant properties of the samples: ferric reducing antioxidant power (FRAP), which measures the sample's ferric reducing power, and 2,2-diphenyl-1-picryhydrazyl (DPPH), which measures the free radical scavenging capacity.

2.5.1 Ferric reducing antioxidant power

The FRAP method is based on the reduction at low pH of a colorless ferric complex (Fe^{3+}–tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}–tripyridyltriazine) by the action of electron-donating antioxidants [28]. Briefly, 90 μL of the extracts was mixed with 270 μL of distilled water, and 2.7 mL of freshly prepared FRAP reagent (25 mL of acetate buffer (0.3 M, pH 3.6), 2.5 mL of TPTZ (tripyridyl triazine) (10 mM) diluted in HCl (40 mM), and 2.5 mL of $FeCl_3{\cdot}6H_2O$ (20 mM)) was added. The absorbance at 595 nm was measured after 5 min. A standard curve was prepared using various concentrations of $FeSO_4 \times 7H_2O$. FRAP values were expressed in millimolar (Fe^{2+}/g of the sample).

2.5.2 DPPH radical scavenging assay

The DPPH assay was performed using 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) [29] and expressed in terms of (a) the amount of extract required to reduce 50% of the DPPH concentration (IC_{50}) and (b) the Trolox equivalents on a dry extract base. Solutions with different concentrations were prepared of the initial extract and of Trolox solution in methanol (0.2 mg/mL). An aliquot of 100 μL of each methanolic solution of extract and Trolox was added to 3.9 mL of a

DPPH methanolic solution (24 μg/mL). The blank sample consisted of 100 μL of methanol added to 3.9 mL of DPPH solution. The absorbance of the extracts was measured at 515 nm and compared with the initial absorbance of the DPPH solution using methanol as blank.

The radical scavenging activity of each sample was calculated by the DPPH inhibition percentage as follows: $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance in the presence of the extract at different concentrations.

The IC_{50} inhibiting concentration, which represents the concentration of a sample necessary to sequester 50% of the DPPH radicals, was obtained by plotting the inhibition percentage against the extract concentration. The scavenging effect on the DPPH radical of the extract was also expressed as the Trolox equivalent antioxidant capacity (TEAC) calculated from the calibration curve with the Trolox solution concentrations and the percentage of scavenging effect on the DPPH radical.

2.6 Pyrolysis experiments

The extracted samples of stem and leaves were powdered in a Retsch MM200 mixer ball mill. The samples (ca. 100 μg) were pyrolyzed in a quartz boat at 550 °C for 10 s using a 5150 CDS apparatus linked to an Agilent GC 7890B coupled to a mass detector system 5977B using electron impact mode (EI at 70 eV). A fused-silica capillary column ZB-1701 (60 m × 0.25 mm i.d. × 0.25-μm film thickness) was used. The gas chromatography conditions and oven program were described in Şen et al. [30]. The pyrolysis products were identified by comparison with Wiley, NIST2014 computer libraries, and by literature, and assigned as derived from lignin, from the S (syringyl), G (guaiacyl), and H (p-hydroxyphenyl) lignin monomeric units, and from carbohydrates. The compounds were calculated using their peak area as a percentage of the total peak area. Total lignin, the lignin monomeric ratios (S/G ratio and H:G:S), and total carbohydrates were calculated according to the sum of peak areas of the corresponding compounds.

3 Results and discussion

3.1 Bark and wood anatomy

The stem bark and wood anatomy of *C. ladanifer* are characterized in Fig. 1a–d and Fig. 2a–d). They show similarities with other genus of Cistaceae and are in accordance with the descriptions made for this species [31, 32].

Microscopy observations on the transverse section of the 3–4-year-old stem of *C. ladanifer* clearly distinguished three parts: pith, wood, and bark (Fig. 1a–d).

Bark (cortex and phloem) and wood (xylem) represent all the tissues outside and inside the vascular cambium, respectively. At this age, the bark still includes primary tissues represented by the epidermis (Ep) with a cuticle and the cortex with large or small thin layered parenchyma cells (cx) (Fig.1a, b). The tangential divisions of cortical cells give rise to the phellogen that produces phellem to the outside and phelloderm to the inside, which together constitute the periderm (Fig. 1c); the periderm is a protective tissue that replaces the epidermis when further growth in diameter takes place. The phloem includes highly thickened fibers in small groups or in tangential rows (mechanical tissue, f) and slightly dilated ray cells (storage and radial conducting tissue, r) (Fig. 1A); the sieve tube elements (conducting tissue) and parenchyma cells (storage tissue) are often difficult to differentiate in cross section; numerous prismatic crystals are present as well as cell contents in parenchyma cells (Fig. 2a).

The xylem is characterized by the presence of growth rings (Fig. 1d, arrows) that is distinct by the presence of marginal parenchyma (Fig. 1b, arrow) and radially flattened and thick-walled fibers. The wood is semi-diffuse to diffuse porous, and the vessels are mostly solitary with alternate inter-vessel pits, thin helical thickenings, and simple perforation (Fig. 2b); the fibers are very thick-walled and pitted (Fig. 2b–d); the axial parenchyma is scarce, paratracheal, and diffuse, and the rays are thin. Prismatic crystals and druses are observed in the parenchyma cells (Fig. 2c, d).

The pith is round (Fig. 1d) with thick-walled and content-filled parenchyma cells.

The accumulation of phenolics in various tissues in the xylem, pith, and cortical parenchyma was also reported by De Micco and Arone [33] in 1-year-old branches of *C. ladanifer,* suggesting a plant defense from animal predation and pathogens.

3.2 Chemical composition

The chemical composition of *C. ladanifer* biomass fractions (stems, branches, leaves, and cysts) and of the residues obtained from two distilleries (CLR1 and CLR2) is given in Table 1. The different CL biomass fractions show distinguishable chemical differences. CL leaves are characterized by a very high extractives content (53.7%) with a significant proportion of lipophilic extractives (26% of total extractives), while stems have much lower extractive content (10.8%) mostly polar (soluble in ethanol and water), which represent 86% of the total extractives. The extractives in cysts and branches fractions were also high, 33.5% and 31.9%, respectively, and polar extractives were dominant, corresponding to 87.6% (cysts) and 80.0% (branches) of the total extractives.

The stem and branches showed similar cell wall lignification, respectively 21.2% and 18.8% of lignin, whereas in leaves and cysts the lignin content was about 15.5%.

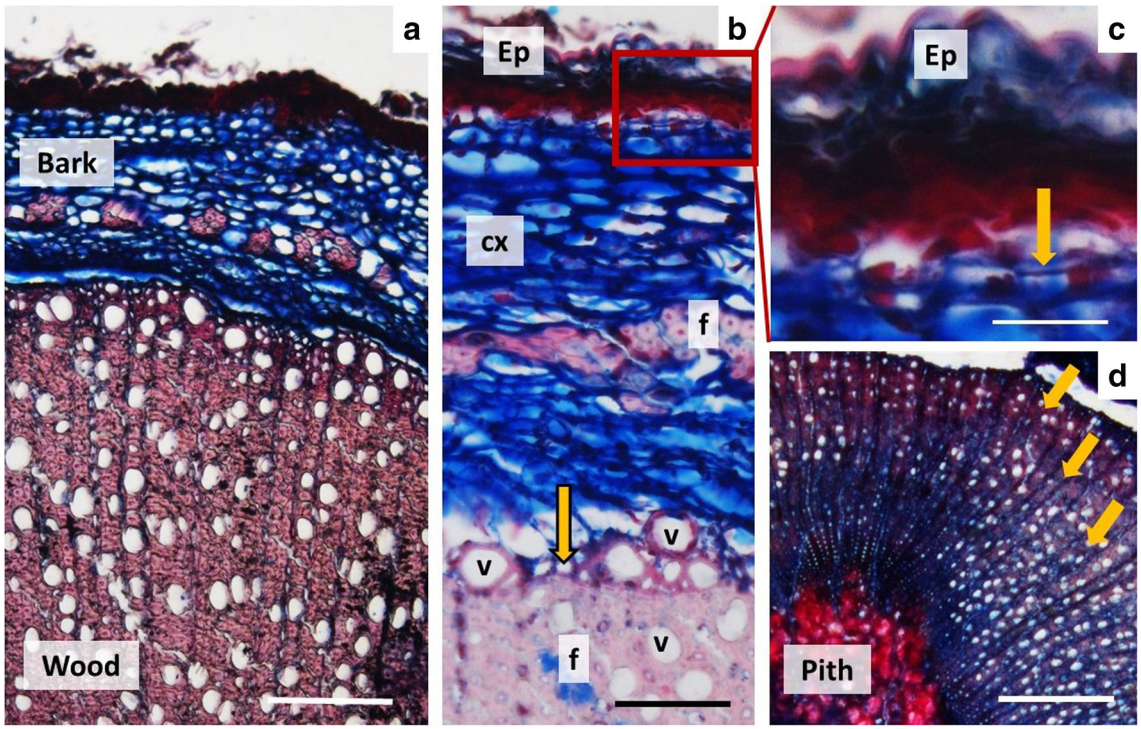

Fig. 1 General structure of a 3-year-old stem of *Cistus ladanifer* in transverse section. **a** Bark and wood. **b** Epidermis (Ep), cortex (cx), thick-walled fibers (f), vessels (v), and scanty marginal parenchyma (arrow). **c** Epidermis with cuticle and cell division of the phellogen (arrow). **d** Pith and wood annual rings (arrows). Scale bar: **a** = 125 μm; **b** = 50 μm; **c** = 100 μm; **d** = 250 μm

The monomeric composition of polysaccharides was similar for all biomass fractions (Table 2). The major monosaccharide was glucose (53.7% of the total monomeric units in stem and 42.8% in leaves), while xylans were the main hemicelluloses (xylose, arabinose, and acetyl groups represented 36.0% and 42.9% of the total monomeric units, respectively in stem and leaves).

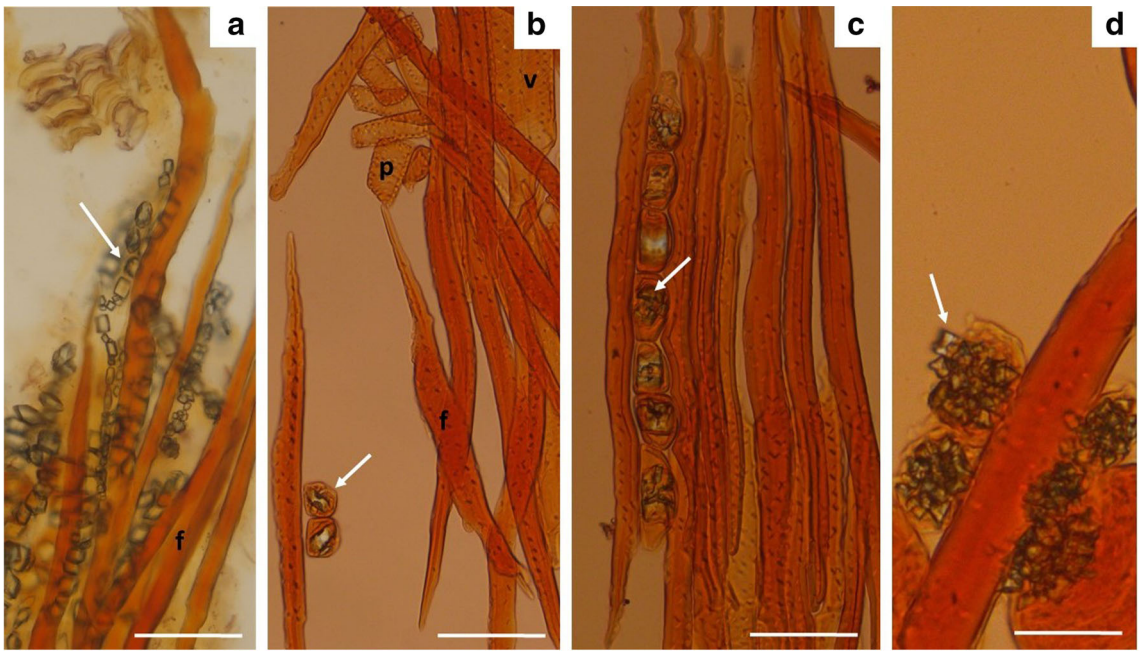

Fig. 2 Individualized cells of bark (**a**) and wood (**b**–**d**). Fibers (f), parenchyma (p), vessels (v), and crystals (arrows). Scale bar: **a**–**c** = 50 μm; **d** = 100 μm

Table 1 Summative composition (% of total dry mass) of *Cistus ladanifer* biomass fractions (stems, branches, leaves, and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries

Chemical composition (% of total dry mass)	Stems	Branches	Leaves	Cysts	CLR 1	CLR 2
Ash	2.15	4.11	5.15	3.89	4.40	5.21
Total extractives	10.81	31.94	53.74	33.53	43.70	39.22
Dichloromethane	1.47	6.40	14.80	4.16	9.09	8.48
Ethanol	6.08	16.54	24.06	12.25	17.16	13.85
Water	3.26	9.00	14.88	17.12	17.45	16.89
Lignin total	21.22	18.57	15.44	15.79	19.29	19.24
Klason lignin	18.23	16.83	13.64	13.99	17.58	17.57
Soluble lignin	2.99	1.74	1.80	1.80	1.71	1.67
Polysaccharides	59.51	40.39	23.30	42.37	29.2	33.27

The chemical composition of the distillery residues (CLR) were on average 4.8% ash, 41.5% total extractives, 19.3% lignin, 17.8% cellulose (with glucose representing 51.7% of total monosaccharides), and 12.3% hemicelluloses (xylose, arabinose, and acetyl groups represent 33.7% of the total monosaccharides).

Galactose or mannose represented values from 1 to 4% of the total content of neutral sugars. Galacturonic acid was also detected in all the samples reaching content from 2.4 to 12.5%.

3.3 Phenolic composition of ethanol–water extracts

The extraction yields of the ethanol/water extracts of the *C. ladanifer* biomass fractions and CLR residues and their total phenolic, flavonoid, and condensed tannin contents are shown in Table 3. The extraction yields depended on the material: the highest yield was in leaves (37.8%) followed by cysts and branches (21.1 and 20.6%) and was lowest in stems (5.8%). These yields are slightly lower than the sum of the soxhlet extraction yields with ethanol (e.g., 6.1% in stem and 24.1% in leaves) and water (3.3% in stem and 14.9% in leaves) and are associated with the extraction conditions used, including the temperature, time of extraction, and solid to liquid ratio. The yields of polar extracts of the CLR residues were 24.1 and 25.7%.

The composition of the extracts also differed among the various biomass fractions of *C. ladanifer*. Table 3 compares the composition of the different extracts with results expressed in milligrams per gram extract and in milligrams per gram biomass (starting material).

The stem extract showed the highest polyphenol content (420.9 mg GAE/g extract); leaf and cyst extracts displayed similar polyphenolic content (303.9–378.8 mg GAE/g extract), and that of the branches extract was lowest (287.1 mg GAE/g extract). When expressed on mg GAE per gram of starting material, which may be of practical interest if this valorization route is envisaged, the difference between biomass components becomes more explicit because of the corresponding extraction yield values. Leaves had the highest content of total phenolics (14.3 mg GAE/g starting material), followed by cysts (6.4 mg GAE/g starting material), branches (5.9 mg GAE/g starting material), and stems (2.4 mg GAE/g starting material).

Comparable phenolic content was reported for ethanol–water extracts of *C. ladanifer* leaves (40.5 mg ferulic acid equivalents/g dry matter) and stem (36.9 mg ferulic acid equivalents/g dry matter) [11] and for ethanolic leaves extracts of *C. salviifolius* and *C. monspeliensis* (49.9 and 56.4 mg GAE/g dry matter, respectively) and for water leaves extracts (54.6 and 37.4 mg GAE/g dry matter respectively) [34].

Regarding flavonoid content (Table 3), the extract of *C. ladanifer* stem was richer (86.8 mg CE/g extract) compared with leaves, cysts, and branches (39.5, 32.3, and 34.4 mg CE/g extract respectively). Higher values were reported for ethanolic and methanolic extracts of *C. ladanifer* leaves (between 61.4 and 64.3 mg rutin/g extract) [35]. When expressed on g CE/g of starting material, the content of flavonoids

Table 2 Monosaccharides and acid composition (% of total neutral monosaccharides) of *Cistus ladanifer* biomass fractions (stems, branches, leaves, and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries

Monosaccharides (% of units)	Stems	Branches	Leaves	Cysts	CLR 1	CLR 2
Arabinose	1.63	4.56	8.09	2.82	5.11	3.77
Xylose	31.92	28.85	22.33	30.71	22.67	27.19
Mannose	3.75	3.69	3.98	2.21	3.74	3.65
Galactose	1.15	2.93	6.67	1.91	3.99	3.18
Glucose	53.66	50.00	42.88	52.48	52.17	51.16
Galacturonic acid	2.40	4.99	12.45	3.52	8.47	6.12
Acetic acid	5.48	4.99	3.59	6.34	3.86	4.94

Table 3 Extraction yield in ethanol/water (50:50) and antioxidant activities of *Cistus ladanifer* biomass fractions (stems, branches, leaves, and cysts) and of the residues (CLR1 and CLR2) obtained from two essential oil distilleries

	Stems	Branches	Leaves	Cysts	CLR 1	CLR 2
Extraction yield (%)	5.8	20.6	37.8	21.1	25.7	24.5
Total phenolic (mg GAE/g extract)	420.87	287.05	378.84	303.86	285.54	270.78
Total phenolic (mg GAE/g biomass)	24.3	59.0	143.1	64.1	73.5	66.4
Tannins (mg CE/g extract)	118.44	34.38	39.54	21.86	22.74	25.91
Tannins (mg CE/g biomass)	6.9	7.1	14.9	4.6	5.9	6.4
Flavonoids (mg CE/g extract)	86.83	57.37	41.76	32.33	33.30	39.00
Flavonoids (mg CE/g biomass)	5.0	11.8	15.8	6.8	8.6	9.6
Antioxidant capacity TEAC (mg Trolox/g biomass)	62.1	159.3	321.5	167.3	159.3	129.7
IC_{50} values (μg extract/mL)	3.2	4.6	4.0	4.7	5.0	6.3
FRAP (mM Fe^{2+}/g extract)	10.13	6.59	8.16	6.59	5.30	5.92
FRAP (mM Fe^{2+}/g biomass)	0.58	1.38	3.08	1.39	1.35	1.45

GAE, gallic acid equivalents; CE: Catechin equivalents

ranged from 1.6 g CE/g dry leaves to 0.5 g CE/g of dry stem. Zidane et al. [11] referred for ethanol–water extract of *C. ladanifer* leaves and stem, respectively 26.0 and 7.9 mg quercetin equivalents/g dry weight. The flavonoid contents in the ethanolic leaves extracts of *C. salviifolius* were reported as 27.8 g CE/100 g dry matter [36] and as 7.0 and 5.3 mg CE/g dry matter, respectively, for ethanolic and water leaves extracts of *C. salviifolius* and *C. monspeliensis* [34].

The proportion of flavonoids in the total phenolics was low among all extracts, e.g., the flavonoids represented only about 20% of the total polyphenol content in stem and branches and 11% in leaves and cysts.

Total phenolics and flavonoid contents are variable in Cistus and depend on the plant tissue evaluated, season, age, and analytical methods [10, 37–40].

Condensed tannins were present in the extracts of all the *C. ladanifer* biomass fractions in variable amounts from 21.9 mg to 118.4 mg CE/g extract respectively for cysts and stems. When expressed in CE/g starting material, the leaves had the largest tannin content (1.5 g CE/g starting material), and the stem the smallest value (0.7 g CE/g starting material), which is in tune with the corresponding extraction yields. Condensed tannin contents for ethanol and water leaves extracts of *C. salviifolius* and *C. monspeliensis* were, respectively, 14.5 and 17.9 mg CE/g dry matter and 16.8 and 22.2 mg CE/g dry matter [34].

The ethanol–water extraction yield and composition of CLR residues are given in Table 3. The extract yield was on average 25.1%, in close agreement with the chemical composition data (Table 1). The total phenolic content (278.2 mg GAE/g extract or 71 g GAE/g dry matter) was in the range of previously published values for whole plant extracts of *C. ladanifer.* Andrade et al. [10] reported 255.2 mg GAE/g extract for ethanol extract of *C. ladanifer.* Barrajón-Catalán et al. [12] reported 22.9 g GAE/100 g dry weight for *C. ladanifer* aqueous extracts. Tomás-Menor et al. [13] found 13.3 g GAE/100 g dry weight in the hydroalcoholic extract of the whole plant of *C. ladanifer*. Nicoletti et al. [41] reported values for plants of different *Citus* species: 40.5 mg GAE/g dry plant for *C. libanotis*, 32.5 mg GAE/g dry plant for *C. villosus*, and 33.2 mg GAE/g dry plant for *C. monspeliensis*. Tomás-Menor et al. [13] reported 21.8 g GAE/100 g dry weight for *C. albidus*, 23.1 g GAE/100 g dry weight for *C. clusii*, and 27.3 g GAE/100 g dry weight for *C. salviifolius*.

Total flavonoid and tannin contents of CLR residues extracts were 36.2 mg CE/g extract (0.9 g CE/100 g dry matter) and 24.3 mg CE/g extract (0.6 g CE/100 g dry matter). Barrajón-Catalán et al. [12] reported 3.0 mg QE/100 mg dry weight for *C. ladanifer* aqueous extracts, and Andrade et al. [10] reported 20.5 QE/g extract and 23.4 QE/g extract, respectively for ethanol and acetone extract. Tomás-Menor et al. [13] reported 0.9 g QE/100 g dry weight for *C. ladanifer*, 1.1 g QE/100 g dry weight for *C. albidus*, 0.7 g QE/100 g dry weight for *C. clusii,* and 1.6 g QE/100 g dry weight for *C. salviifolius*.

3.4 Antioxidant activity

The results for the antioxidant potential of the ethanol–water extracts of the *C. ladanifer* fractions using DPPH and FRAP assays are summarized in Table 3.

The DPPH radical scavenging activity is reported in terms of IC_{50} as well as of the Trolox equivalents (TEAC) on a starting material basis (mg TEAC g^{-1} of starting material). These extracts revealed high efficiency as a free radical scavenger, with an IC_{50} value between 3.2 and 4.7 μg/mL when compared with Trolox (IC_{50} of 2.7 μg/mL). The IC_{50} of the CLR extracts also indicates high antioxidant activity (IC_{50} of

6.3 and 5.0 μg/mL), comparing very favorably with the IC_{50} values of well-known antioxidant standards such as catechin (5.4 μg/mL) and Trolox (2.7 μg/mL), the latter is considered to have excellent antioxidant activity.

Substantially low values of IC_{50} were reported for aqueous extracts of *C. salviifolius* leaves (1.3–6.5 μg/mL) and *C. monspeliensis* leaves (1.2 μg/mL) [34, 42]. Zidane et al. [11] reported also high scavenging ability of DPPH radicals for methanolic extracts of different plant parts of *C. ladanifer* and *C. libanotis*.

All the extracts showed also strong ferric ion reducing activities that ranged from 6.6 to 10. 1 mM Fe^{2+}/g extract (respectively for branches and stems). Expressed on the starting material, the leaves extract showed the highest ferric ion reducing capacities (308.2 mM Fe^{2+}/g) and stem extracts the lowest (58.6 mM Fe^{2+}/g). The CLR extracts also have strong ferric ion reducing capacities (5.9 and 5.3 mM Fe^{2+}/g extract, respectively, 145 and 135 mM Fe^{2+}/g). The results obtained for *C. ladanifer* are comparable with those previously reported as 117.7 mmol Fe^{2+}/100 g dry weight and 179.1 mmol Fe^{2+}/100 g dry weight for *C. populifolius* [12] and 318.8 mmol Fe^{2+}/100 g dry weight for *C. salviifolius* aqueous extracts [13].

3.5 Lignin composition

Figure 3 shows the pyrograms obtained for the stem and leaves of *C. ladanifer*, and Table 4 lists the lignin-derived compounds obtained by pyrolysis, including CLR1 sample previously described [43] (classified as derived from the H, G, and S lignin units). From the total of 82 pyrolysis products that were identified in the pyrograms (data not shown), 29, 28, and 27 peaks were identified as originating from lignin-derived compounds for the stem, leaves, and CLR, respectively.

The pyrograms (Fig. 3) show that the main lignin-derived compounds obtained from pyrolysis of the stem were 4-vinylsyringol (peak 65, S), *trans* 4-propenylsyringol (peak 73, S), and syringaldehyde (peak 74, S) while those from leaves were 2,3-dihydrobenzofuran (H) and 4-vinylguaiacol (G) (peak 48/49 overlapped).

There was a clear difference in the lignin composition of stem and leaves: the H:G:S ratio was 1:25:50 for stem lignin and 1:1.4:1 for leaves lignin (Table 4). Stem lignin is an SG-lignin composed predominantly of S units (65.8% of units) with an only very minor amount of H units, whereas lignin in leaves is an HSG lignin constituted mainly by G units (41.2% of units) but with substantial and equal amounts of S and H units (29.4%). CLR presented S/G ratio similar to that of the leaves (0.9 vs 0.7), evidencing once again the strong presence of leaves in these type of residues (Table 4).

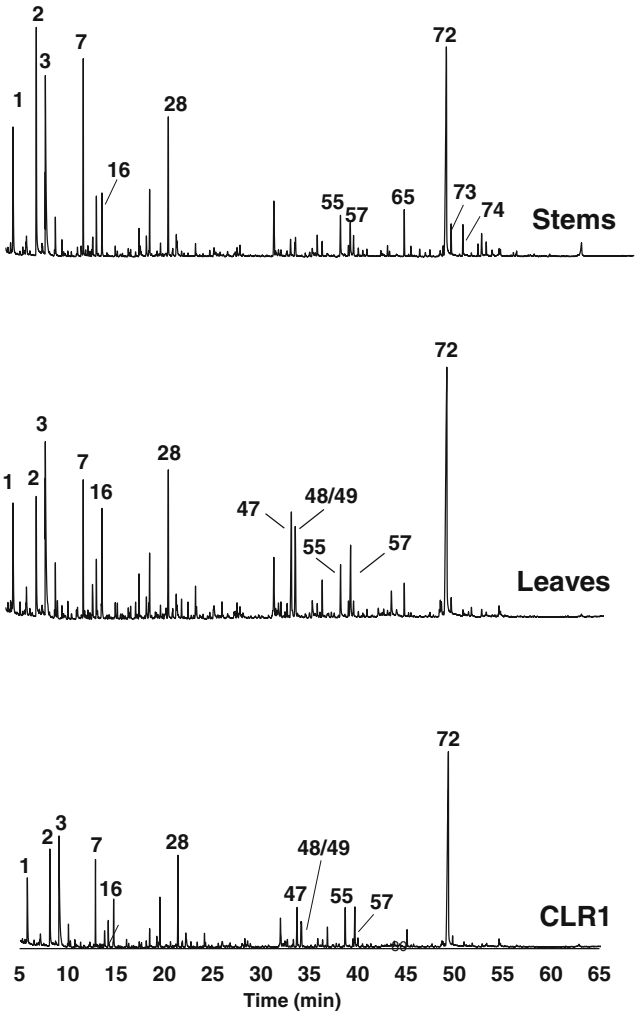

Fig. 3 Py-GC/MS chromatograms of stems, leaves, and CLR1 of *C. ladanifer*. Main peaks: (1) 2-oxo-propanal, (2) hydroxyacetaldehyde, (3) acetic acid (7) 3-hydroxypropanal (16) furfural, (28) 4-hydroxy-5,6-dihydro-2H-pyran-2-one, (47) 1,5-anhydro-arabinofuranose, (48) 2,3-dihydrobenzofuran, (49) 4-vinylguaiacol, (55) 2-hydroxymethyl-5-hydroxy-2,3-dihydro-4H-pyran-4-one, (57) similar to 1,5-Anhydro-arabinofuranose, (65) 4-vinylsyringol, (72) 1,6-anhydro-β-D-glucopyranose, (73) trans 4-propenylsyringol, (74) syringaldehyde

It has been shown that lignin composition varies between species and between tissues in a plant [44]. Micco and Aronne [33] found a predominance of S units in xylem and of G units in the pith of 1-year-old branches of *C. ladanifer*. Lignin composition also differs between the different morphological parts of the banana plant, e.g., leaf sheaths (H:G:S of 1:2:0.5), leaf blades (1:9.3:6.3), and floral stalks (1:1.6:1) [45]. The study of lignin in xylem, phloem, and cork of *Quercus suber* showed that the different tissues presented different H:G:S compositions: lignin from xylem is enriched in S units (1:45:55), the lignin from phloem has less S and more G units (1:58:41) and the cork lignin is enriched in G units (2:85:13) [46].

The S/G ratios of lignin in *C. ladanifer* stem and leaves were 2.2 and 0.7, respectively. The S/G ratio is an important

Table 4 Identification of the lignin-derived pyrolysis products (as % of the pyrogram peak areas) in stems and leaves of *Cistus ladanifer*

Lignin products	Retention time	% of the total area		
		Stems	Leaves	CLR1[a]
Syringol derivatives (S)				
Syringol	36.38	0.8	0.4	0.4
4-Methylsyringol	40.02	0.8	0.3	0.6
4-Ethylsyringol	42.89	0.1	0.2	0.1
4-Vinylsyringol	45.11	1.5	0.8	0.8
4-Allylsyringol	45.78	0.3	0.1	0.1
cis 4-propenylsyringol	47.68	0.2	0.1	0.2
4-Propinylsyringol	49.02	0.3	0.1	0.1
trans 4-propenylsyringol	49.82	1.1	0.5	0.4
Syringaldehyde	51.01	1	0.1	0.2
Homosyringaldehyde	52.57	0.4	n.d.	n.d
Acetosyringone	53.34	0.5	0.1	0.2
Syringylacetone	54.74	0.2	0.2	0.2
trans sinapaldehyde	62.90	1	0.1	0.2
Guaiacol derivatives (G)				
Guaiacol	24.16[#]	0.2	0.6	0.3
4-Methylguaiacol	28.32	0.4	0.4	0.6
4-Ethylguaiacol	31.82	0.04	n.d.	0.05
4-Vinylguaiacol	34.15[#]	0.7	1.6	1.2
Eugenol	35.16	0.1	0.1	0.1
trans isoeugenol	39.50	0.4	0.5	0.5
Vanillin	40.50	0.3	0.2	0.2
1-(4-Hydroxy-3-methoxyphenyl)-propyne	40.95	0.2	0.1	0.1
1-(4-Hydroxy-3-methoxyphenyl)-propyne	41.36	0.3	0.2	0.2
Homovanillin	42.76	0.2	n.d.	0.1
Acetoguaiacone	43.64	0.2	0.2	0.1
Guaiacylacetone	45.55	0.1	0.1	0.1
trans coniferyl alcohol	47.22[#]	0.1	n.d.	n.d
Guaiacyl vinyl ketone	47.22[#]	0.1	n.d.	n.d
trans coniferyl alcohol	53.93	0.1	n.d.	n.d
trans coniferaldehyde	54.65	0.3	0.2	0.6
p-hydroxyphenyl derivatives (H)				
Phenol	23.38	0.1	0.4	0.2
o-Cresol	25.33	n.d.	0.4	n.d
p-Cresol	26.79	n.d.	0.4	0.2
m-Cresol	26.87	n.d.	0.1	n.d
2,3-Dihydrobenzofuran	34.15[#]	n.d.	1.6	n.d
2,3-Dimethyl-phenol	28.72	n.d.	0.1	n.d
Not determined lignin source (NDL)				
Toluene	10.26	n.d.	0.4	0.3
Styrene	14.29	n.d.	0.2	0.1
S		8.0	3.1	i3.5
G		3.7	4.3	4.0
H		0.1	2.9	0.5
S/G		2.2	0.7	0.9
H:G:S		1:25:50	1:1.4:1	1:1.1:0.1
NDL		n.d.	0.7	0.4

[#] Compounds overlapped; *n.d.*, not detected

[a] Some values are published in Alves-Ferreira et al. (2019)

parameter for the chemical evaluation of lignin especially regarding pulping potential due to the higher reactivity of the S units [44]. Thus, the stem of *Cistus* biomass is more suitable for delignification processes, while the leaves may not be very appropriate for this type of chemical approach. Although the industrial valorization of lignins is still rare, their conversion into chemicals will positively influence the economic viability of lignocellulosic biorefineries [47].

3.6 Phenolic profile of soxhlet extracts

The phenolic profiles obtained by CZE at 280 nm for the ethanolic and water soxhlet extractives are shown in Figs. 4 and 5.

From the electropherograms of the extractives solubilized in ethanol shown in Figs. 4 and 5, it can be seen that the phenolic profiles are highly complex and in agreement with

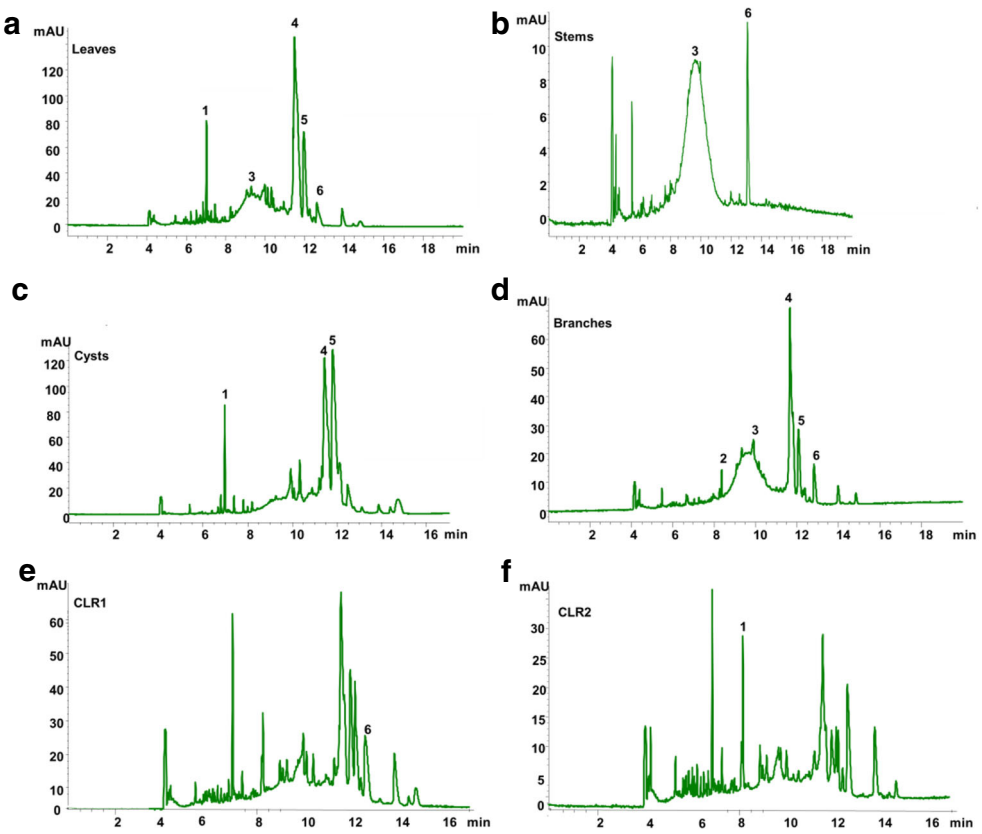

Fig. 4 Electropherograms (280 nm) showing the phenolic profile of ethanolic extracts of *Cistus* fractions (leaves, stems, cysts, branches, and distillery residues (CLR1, CLR2)). Peak identification: (1) apigenin, (2) isoquercetin, (3) gallocatechins, (4) unidentified compound, (5) unidentified compound, (6) Gallic acid. See text for CZE for phenolics separation

the results obtained for the total phenolics content. In Fig. 4a–f, it is possible to observe the presence of the flavonoid apigenin (peak 1) in the samples of cysts, leaves, and CLR2. A band showing the presence of gallocatechins (peak 3) and gallic acid (peak 6) confirms the presence of tannins which were the main polyphenols in the stem extractives. In fact, these compounds were also detected in the leaves and branches extractives. In addition, gallic acid was also found in CLR1 samples. Branch extract was the only sample where the presence of isoquercetin (peak 2) was detected. Peaks 4 and 5 in the samples of cysts, leaves, and branches, could not be identified. Other compounds such as hydroxybenzoic acid and vanillic acid were also found in the ethanolic extractives at 200 nm (data not shown).

For the water extractives, the phenolic profile also shows some complexity, but only gallic acid could be identified with the available standards. As an example, the electropherograms obtained for leaves and CLR1 can be seen in Fig. 5.

Gallic acid, gallocatechin, rutin, apigenin, and vanillic acid from cistus extracts were already determined in previous studies [12, 13, 30, 48–50]. In fact, *C. ladanifer* extracts were characterized in several works, since this plant is traditionally recognized by its medicinal properties. The studies on the phenolic composition of *C. ladanifer* extracts indicate mainly the presence of gallotannins, flavonoids, and phenolic acids. However, ellagic acid derivatives were the most abundant group determined by Barros et al. [48] in aqueous methanolic extracts of CL, with punicalagin gallates as the main compounds. Tomás-Menor et al. [13] reported differences between aqueous and hydroalcoholic extracts of *C. ladanifer*, with this latter presenting flavonoid derivatives such as apigenin methylether and kaempferol dimethylether. Other compounds such as quercetins, kaempferol derivatives, 49-methyl-

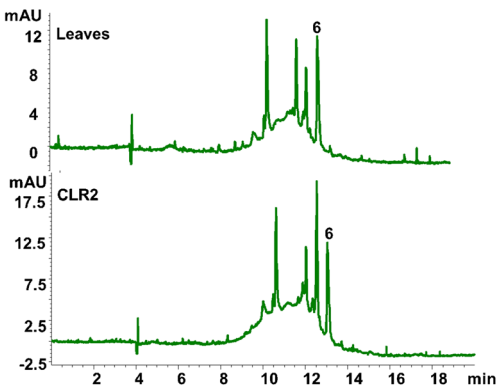

Fig. 5 Electropherograms (280 nm) showing the phenolic profile of the aqueous extracts of *Cistus* fractions exemplified by two samples (leaves and distillery residues (CLR1)). Peak identification: (6) gallic acid. See text for CZE for phenolics separation

apigenin, 7-methyl-apigenin, quinic acid, 3-β-D-glucoside, and cornusiin B ellagitannins as punicalin, punicalagin, and gallagic acid were previously reported in different extracts of cistus [13, 48–50].

Various studies were also conducted in order to know the profile of the volatile components present in the lipophilic extracts of *C. ladanifer*. For dichloromethane extracts from leaves of CL, 2,2,6-trimethylcyclohexanone, acetophenone, and 2-phenylethanol were the main compounds detected, being these molecules extremely aromatic [51]. Other volatiles as α-pinene, camphene, camphor, fenchone, and verbenone from shoots of CL extracted with hexane were also identified [52]. Morales-Soto et al. [53] described the volatile profile of *Cistus* plants and determined 51 compounds for samples of *C. ladanifer*, with monoterpenes and sesquiterpenes as the main families of lipophilic compounds.

Thus, the results of this work showed that a high quantity of different constituents was identified from soxhlet ethanolic extracts, but also aqueous extracts revealed to have a complex matrix, although only gallic acid could be identified by CZE.

4 Conclusions

The biomass components of the *C. ladanifer* plants, e.g., stem, branches, leaves, and cysts show different chemical composition, mostly regarding the content of extractives which are particularly high in leaves, e.g., fivefold the content in stems, while lignin content is highest in stems and branches. Lignin composition also shows differences with more S units in stem lignin and important amounts of H units in leaves lignin. The distillery residues (CLR) have a composition resulting from the combination of the different biomass fractions. An important chemical feature of *C. ladanifer* components and of their distillery residues (CLR) is the high content of extractives, especially of polar extractives that were rich in phenolics, namely flavonoids and tannins, which showed high antioxidant activity.

The high extractive contents of *C. ladanifer* point out to the potential use of this species as a source of phytochemicals by applying extraction procedures. The remaining lignocellulosic material after extraction may be directed towards potential lignin and carbohydrates applications.

The use of *C. ladanifer* biomass for an extractive-lignocellulosic-based biorefinery therefore represents a potential valorization that may contribute to additional revenue for the present essential oil distilleries.

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