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Bioproducts from forest biomass II. Bioactive compounds from the steam-distillation by-products of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. wastes

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ABSTRACT

Obtaining essential oils and hydrolates from underutilized biomass is an economic and sustainable way for production of these high added-value bioproducts. However, this process still generates large amounts of residues as the by-products obtained during distillation, which can be a concern for the environment, but also adequate substrates for other applications. Considering this fact, the waste distilled by-products remaining after steam-distillation of underutilized biomass from *Cupressus lusitanica* and *Cistus ladanifer*, were evaluated as a natural source of other high value products with biological activities, namely, phenolic compounds. Thus, the remaining extracted solid residues (ESRs) were characterized and subject to further treatments by ultrasound-assisted extraction (UAE) with ethanol and 70 % acetone, in order to prepare phenolic-rich extracts thereof: ESRs (EtOH) and ESRs(70 % Ace). Together with the distiller condensation waters (DCWs), these extracts were characterized for their phenolic content (total phenols, tannins and flavonoids). Their antioxidant activity was also evaluated by different methodologies. The phenolic profile of DCWs, ESRs(EtOH) and ESRs(70 % Ace) from both waste species was obtained by capillary zone electrophoresis (CZE) and phenolic compounds were tentatively identified. Results obtained for *C. lusitanica* biomass are here disclosed for the first time. Generally, all samples revealed to be rich in phenolic compounds, being *C. ladanifer* biomass the one with higher phenolic content. DCWs presented values of 140 mgGAE/g for *C. lusitanica* and 210 mg GAE/g for *C. ladanifer*, from which ca. 60 % were tannins. Extracts obtained with 70 % acetone were the ones with the highest results, except for the antioxidant activity by xanthine oxidase and superoxide inhibition, which was higher in DCWs. Catechins were the major compounds found for both species, but gallocatechins and gallic acid were only identified in *C. ladanifer*. Hydroxycinnamic acid derivatives and salicylic acid were also identified in *C. ladanifer*, partly justifying the anti-inflammatory effect referred for this species.

1. Introduction

Following the Renewable Energy Directive (Directive 2009/28/EC), the Roadmap 2050 (2015) and the Paris Agreement to reduce greenhouse gas emissions by at least 40 % by 2030 (2015), the use of biomass as a renewable source of energy has been significantly encouraged, and forest residues such as bushes and aerial parts of trees, which play an important role in forest management, have been used mainly for fuel (Puy et al., 2011). However, the use of these bioresources for producing high added value products is becoming more and more important in the

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context of sustainability and bioeconomy, as biomass of natural origin can be recovered in biorefineries with environmental, economic and social benefits (Budzianowski, 2017; Ali et al., 2015). In the scope of a policy of valorisation of renewable energy sources, and in the context of the valorisation of biomass according to the existing national potential, the Portuguese National Plan for the Promotion of Biorefineries (PCM, 2017) was launched, which reinforces the valorisation of the renewable energy sources through the sustainable use of biomass not only for energy, but also for various economic sectors. Generally, biorefineries are primarily energy-based, i.e. the plant is optimised primarily to generate bioenergy products from biomass, (namely biofuels, electricity and heat), while generating co-products that may be precursors of products of higher added value for non-energy applications. However, there are biorefineries that are optimised to generate (in mass percentage) mainly bioproducts, namely, biomolecules, intermediate chemicals, proteins, bioactive substances, etc (Cho et al., 2020; Mahmood et al., 2019; Chirat, 2017).

In our previous study, (Tavares et al., 2020), the potential for obtaining essential oils and hydrolates from the underutilised biomass of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L., their chemical characterisation and several *in vitro* bioactivities were evaluated. Numerous biological activities have been attributed to the essential oils and these are value-added products from biomass that can be readily used in the perfume/cosmetic industry or as bioblocks for different other industries (Tavares et al., 2020). Nevertheless, the yield of distillation is considerably small and the process for obtaining essential oils and hydrolates from the biomass still generates large amounts of residue as the by-product obtained during distillation, which is of growing concern for the environment if not properly managed. In the present study, the by-products remaining after removing the essential oil through steam-distillation, namely, distiller condensation waters and the extracted solid residues were evaluated as natural sources of other high value products with biological activities, namely, phenolic compounds, which are valuable extractives from biomass (Volf and Popa, 2018), and can be mainly used as antioxidants for different industries, before an ultimate energy application of the solid residues. Identification of new sources of natural antioxidants is a priority for example, for the food and feed industries, as the safety of the widely used authorized preservatives, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) is very controversial (Wollinger et al., 2016). Thus, the aim of this work was to test the potential of the remaining residues from steam-distillation as source of antioxidant compounds and broaden the utilization of these biomasses. Fig. 1 illustrates the complete valorisation potential of these biomasses, highlighting the procedure described in the present study.

2. Materials and methods

2.1. Plant material and steam-distillation by-products

Cupressus lusitanica Mill. and *Cistus ladanifer* L. underutilized biomasses (aerial parts) were collected separately for obtaining essential oils and hydrolates by steam-distillation, which was performed at a semi-industrial scale using a stainless-steel distiller (1100 L, Vieirinox®, Aveiro, Portugal) at SILVAPOR premises, as described in Tavares et al. (2020). Circa 100 Kg of aerial parts were subject to steam-distillation to get an average essential oil yield <0.3 % for *Cupressus* and <0.04 % for *Cistus*, and 20 L of hydrolate from each biomass (Tavares et al., 2020). After steam-distillation of each of these biomasses, samples of the remaining extracted solid residues (ESRs) were collected and taken to the lab for further sequential ultrasound assisted extraction (UAE), which is a simple and efficient extraction method that prevents possible chemical degradation of the targeted compounds (Ghafoor et al., 2009), using ethanol (EtOH) and 70 % acetone (70 % Ace), and characterization of the corresponding extracts. Also, 1.5 L of the distiller condensation waters (DCWs) were obtained from each biomass and distillation assay, collected and freeze-dried for further analysis.

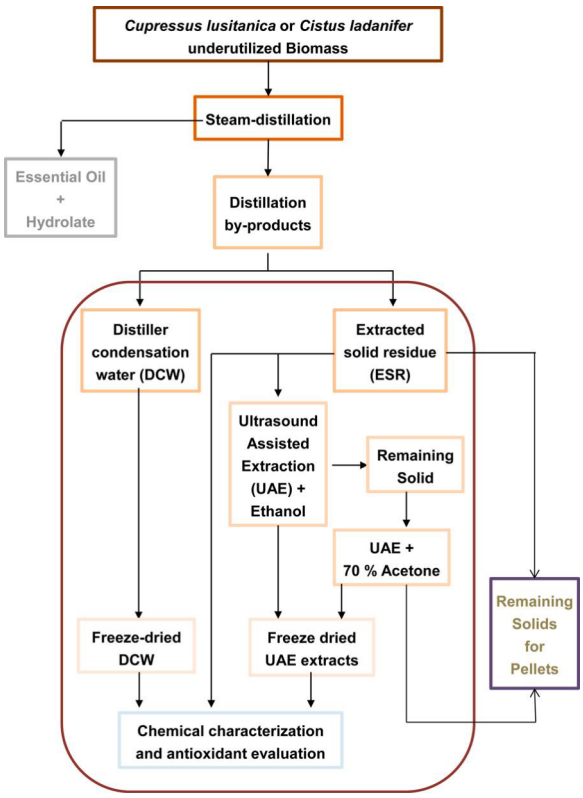

Fig. 1. Illustrative scheme for the valorisation of *Cupressus lusitanica* or *Cistus ladanifer* underutilized biomass, highlighting the present study (round corner rectangle).

2.2. Characterization of the extracted solid residues (ESRs)

Representative samples of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. ESRs obtained after steam-distillation were air-dried for one week at open air conditions, then chemically characterized for their moisture, ash, carbohydrate, Klason lignin, soluble lignin and protein content. After milling to a particle size smaller than 0.5 mm, the moisture content was determined by oven-drying at 105 °C to constant weight. Ash content was determined at 550 °C using NREL/TP-510-42622 protocol (Sluiter et al., 2008). The quantification of macromolecular compounds was determined by sequential quantitative acid hydrolysis with 72 % (w/w) H_2SO_4 and 4 % (w/w) H_2SO_4 following a method based on NREL/TP-510-42618 protocol (Sluiter et al., 2012). The amounts of glucan, xylan, arabinan, galactan and mannan were calculated based on the concentrations of sugars in hydrolysates obtained after quantitative acid hydrolysis. An HPLC (Agilent, Germany) was used, equipped with RI detector and an Aminex HPX-87 P column (Bio-Rad, Hercules, CA, USA) operating at 80 °C, in combination with a microguard CarboP column (Bio-Rad), and using water as the mobile phase at the flow rate 0.5 mL/min. The acid-insoluble residue was considered as Klason lignin, after correction for ash. The acid-soluble lignin was determined in the filtrate of sugars in hydrolysates by UV spectroscopy at 206 nm using 110 L / (g cm) as absorptivity (extinction coefficient) (TAPPI UM-250, 1991). The determination of protein was carried out according to the Kjeldahl method (AOAC, 1975) using the N × 6.25 conversion factor.

2.3. Preparation of phenolic-rich extracts from extracted solid residues

The ESRs obtained after steam-distillation from either *C. lusitanica*

and *C. ladanifer* were mixed with ethanol at a solid:liquid ratio of 1:20 and subjected to ultrasound-assisted extraction (UAE) at 30 °C for 30 min, using a Transsonic T700 sonifier (320 W, 35 kHz) (Elma GmbH & Co, Germany), according to a previously used UAE method for phenolics from biomass (Roseiro et al., 2013a). Extracts were then filtered through filter paper (Whatman nº. 1), concentrated under vacuum at 45 °C in a Rotavapor R-210 BUCHI (with vacuum controller V-850 and heating Bath B-491, also from BUCHI) to obtain the ethanolic extract. This procedure was repeated 3 times. The remaining UAE solids were further extracted with 70 % acetone at a solid:liquid ratio of 1:20 using the same procedure, and the acetone extracts were pooled, concentrated under vacuum and freeze-dried at -56 °C in a Heto Power Dry LL3000, Thermo Scientific. Fig. 2 shows a schematic drawing of the procedure. Remaining solids from both UAEs solvent systems of each biomass were oven dried at 45 °C for one week, and stored for future trials to produce pellets.

2.4. Phenolic composition from distiller condensation waters (DCWs) and ultrasound-assisted extracts (UAEs) of extracted solid residues (ESRs)

2.4.1. Total phenolics, tannins and non-tannins content

Total phenolics were determined in the DCWs and all the UAEs extracts for both *C. lusitanica* and *C. ladanifer* biomass residues, by the Folin–Ciocalteu colorimetric method according to the procedure described in Roseiro et al. (2013b), adapted to a microplate format using spectrophotometric detection and microtiter 96-well plates. Briefly, reconstituted samples (1 mg/mL) of distiller condensation waters and UAE extracts (0.1 mL; or water for blank) were mixed with 0.4 mL distilled water, 1/1 (v/v) diluted Folin–Ciocalteu reagent (0.25 mL) and 20 % m/v Na_2CO_3.10 H_2O (1.25 mL). Aliquots of 200 µL were placed in each microplate well. Absorbance was measured at 725 nm on a microplate reader (Multiscan GO, ThermoFischer Sc.). A calibration curve of gallic acid was prepared. Tannins content was determined based on the same methodology as above (Roseiro et al., 2013b), after removal of tannins by their adsorption on an insoluble matrix (polyvinylpolypyrrolidone, PVPP). The non-adsorbed phenolics (non-tannins) in the supernatant were transferred into the microplate wells and determined as previously described. Calculated values were subtracted from total phenolics content to obtain the total tannins content. Results were expressed as mg gallic acid equivalent (GAE) / g of extract.

2.4.2. Flavonoids content

Flavonoids content were determined in the DCWs and all the UAEs extracts for both species according to Miguel et al. (2014) with some modifications. Briefly, 0.25 mL of 2 % aluminium chloride-ethanol solution was added to 0.25 mL of reconstituted sample or standard in a test tube. After 1 h at room temperature, absorbance was measured at 420 nm using a UV–vis Shimadzu UV-160A spectrophotometer. Quercetin was used as a standard for the calibration curve. Results were expressed as mg quercetin equivalent (QE) / g of extract.

2.4.3. Phenolic profile by Capillary Zone Electrophoresis (CZE)

Phenolic profile of DCWs and all the UAEs extracts was obtained by capillary zone electrophoresis (CZE) using an Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (DAD), as described in Roseiro et al., 2013b. Electropherograms (e-grams) were recorded at 200 and 280 nm, and phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) with data from authentic standards run under the same conditions and stored in library.

2.5. Antioxidant activity

The antioxidant activity of DCWs and all the UAE extracts for both

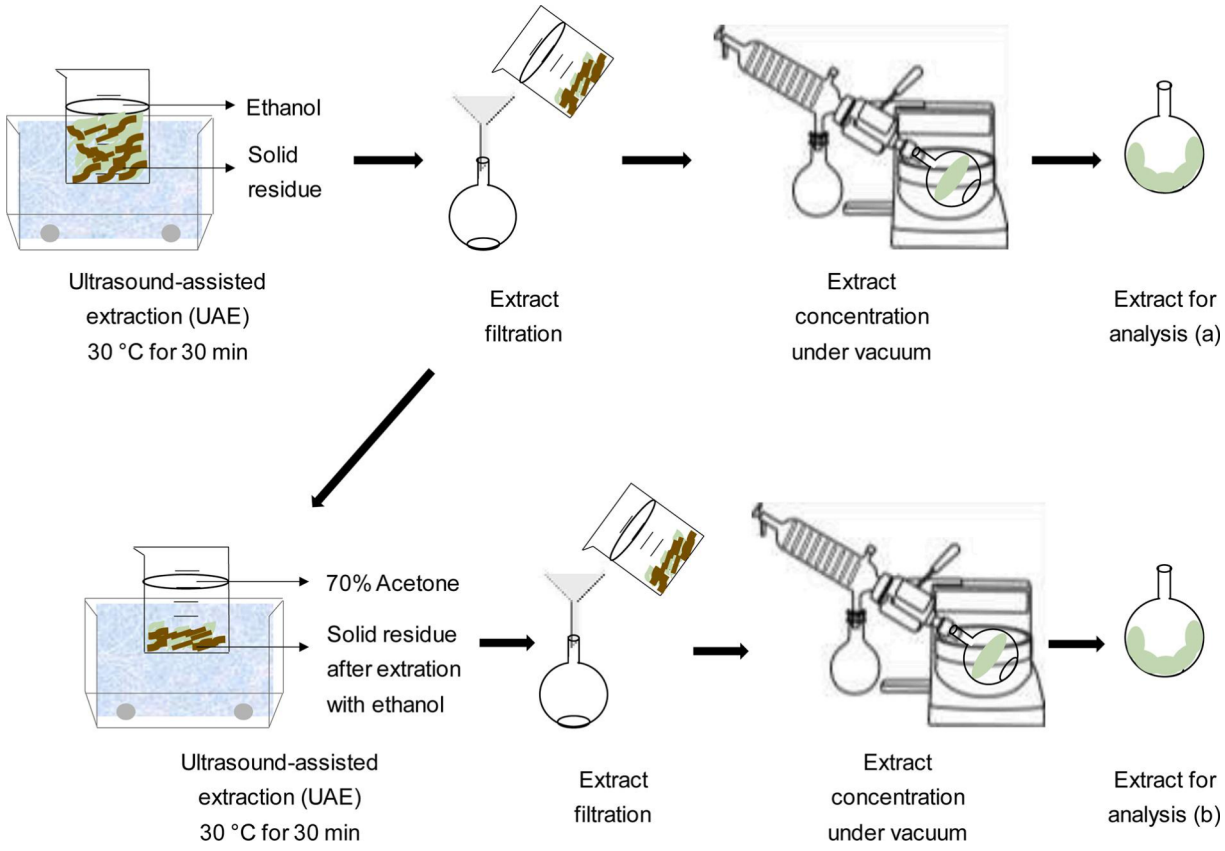

Fig. 2. Schematic representation of the sequential ultrasound-assisted extraction (UAE) from the extracted solid residues (ESRs), using ethanol [extract for analysis (a)] followed by 70 % acetone [extract for analysis (b)].

C. lusitanica and *C. ladanifer* biomass by-products obtained by steam-distillation was determined using different methodologies as referred previously in Tavares et al. (2020) and summarized below. These methodologies were chosen according to some of the standardized antioxidant method criteria, namely, for being simple, rapid and reproducible with chemicals and instrumentation readily available using methods with a defined endpoint and chemical mechanism, for both hydrophilic and lipophilic antioxidants, and being representative of biomolecules.

2.5.1. ABTS cation radical decolourisation assay

The ABTS radical scavenging was carried out as reported by Re et al. (1999) adapted to a microplate format using spectrophotometric detection (Multiscan GO, ThermoFischer Sc.) and microtiter 96-well plates. Aliquots of the reconstituted DCWs and UAEs extracts (30 μL) were added to the radical solution (3 mL) and 200 μL of each and placed in each microplate well. Trolox ((±)-6-Hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic-acid) was used as standard and results were expressed as Trolox equivalent antioxidant capacity (TEAC).

2.5.2. Inhibition of superoxide anion radical formation

Scavenging ability of superoxide anion radical was evaluated according to Soares (1996) with some modifications. In brief, reconstituted samples (60 μL) were used and the reaction mixture was incubated at room temperature for 10 min and the absorbance reading was performed at 560 nm in a UV/VIS spectrophotometer. Ascorbic acid was used as standard and results were expressed as ascorbic acid equivalent (AAE).

2.5.3. Inhibition of xanthine oxidase

Xanthine oxidase inhibiting activity followed the Umamaheswari et al. (2013) method using allopurinol as standard and 50 μL of the reconstituted samples. The assay mixture was incubated for 30 min, after which the reaction was stopped and the absorbance was measured at 290 nm in an UV/VIS spectrophotometer. Results were expressed as allopurinol equivalent (AE).

2.5.4. Chelating metal ions

Chelating of ferrous ions by the reconstituted DCWs and UAEs samples (200 μL) was evaluated according to Wang et al. (2004) method. EDTA (ethylenediamine tetra-acetic acid) was used as standard and results were expressed as EDTA equivalent (EDTAE).

2.6. Statistical analysis

All analytical determinations were carried out in quadruplicate and results are presented as mean values with their corresponding standard deviations.

3. Results and discussion

3.1. Chemical composition of the extracted solid residues

Literature data for *Cupressus lusitanica* other than essential oil are very scarce, particularly when compared with literature for *Cistus ladanifer*. To the best of our knowledge, there are no published results concerning the chemical composition of *C. lusitanica* aerial parts neither for other *Cupressus* trees. Table 1 compares the results here obtained with results in literature for *Juniperus* spp, which are the same family (Cupressaceae) and for *Pinus radiata* and *Picea abies* wood, both the same order as *Cupressus* (Pinales) and also softwood trees. Table 2 shows the composition of *C. ladanifer* aerial parts used in the present study and compares it with literature results. It can be observed from Table 1 that the composition of the extracted solid residue for *C. lusitanica* differs largely, which would be expected, considering that, not only this has suffered an extraction by steam-distillation, but also its native biomass consisted on leaves, small branches and globular seed cones from the tree top, and not the wood from the trunk of the tree. Only the cellulose value was similar to the one for *Juniperus sibirica* needles, but conversely, Klason lignin was within the range of values found for the other species wood.

Table 2 shows results from the present study for *C. ladanifer* and from literature, including the ones disclosed by Alves-Ferreira et al. (2019a); (2019b), and (2017), which are within the same broad research study. Results show that Klason lignin (37 % w/w) was much higher in the present study than in the others, particularly when compared to results from Alves-Ferreira et al. (2019b), and (2017), and also higher than the carbohydrate content (26.2 % w/w). The later agree with the ones previously determined by Alves-Ferreira et al. (2019b), and (2017). Also, for other studies, the composition of the starting material used was different from the one here described. Alves-Ferreira et al. (2019a) and Fernandes et al. (2018) used the extracted solid residue from steam distillation and the raw material, respectively, after being subject to Soxhlet extraction with several solvents, and obtained lower results for Klason lignin (29 % and 32 % w/w) but higher results for carbohydrate content (47 % and 41 % w/w), respectively, when compared to our study, nevertheless, both data were within the same range. However, Carrión-Prieto et al. (2017) and Ferro et al. (2015), which used the original raw material, obtained values of 25 % and 16 % w/w for lignin and 65.2 % and 46 % w/w for carbohydrate, respectively, showing their heterogeneity. Despite all studies refer to the aerial parts, the fact that one has more leaves in its composition and the other more branches,

Table 1
Chemical composition of the extracted solid residue (ESR) from distilled aerial parts of *Cupressus lusitanica* and comparison with other species of the same family (Cupressaceae) and order (Pinales).

Plant part	*Cupressus lusitanica*	*Juniperus sibirica*	*Juniperus communis*		*Picea abies*	*Pinus radiata*		
	ESR	Needles	Wood	Wood	Wood	Wood	Wood	Wood
Cellulose								
Glucan	**14.6**	18.1–20.9	38.0–41.0	61.9	44.0	45.3	31.1–42.5	44.9–54.1
Hemicellulose	**17.7**			37.9	23.3	22.2	21.4–26.0	6.1–11.1
Xylan	**7.5**			11.9	6.0	6.4		
Arabinan	**2.5**			1.4	2.0	1.5		
Galactan	**3.3**			7.5	2.3	2.1		
Manann	**4.4**			17.1	13.0	12.2		
Klason lignin	**39.0**	15.2–19.9	30.0–32.0	30.1	27.5	26.8	29.4–39.0	26.3–30.0
Soluble lignin	**2.5**							
Protein	**6.6**							
Ash	**5.7**				1.6	0.2		
Others (by difference)	**13.9**							
Reference	**Present study**	Artemkina et al. (2016)	Bogolitsyn et al. (2015)	Hänninen et al. (2012)	Sassner et al. (2008)	Araque et al. (2008)	Berrocal et al. (2004)	Uprichard (1971)

Table 2
Chemical composition of the extracted solid residue (ESR) from distilled aerial parts of *Cistus ladanifer* obtained in the present work and comparison with previous studies in literature.

Chemical composition (% w/w)							
	Aerial parts						
Plant part	ESR	ESR	ESR	ESR(Sx)	RM(Sx)	RM	RM
Cellulose							
Glucan	**10.6**	17.8	16.1	27.8	26.6	55.0	34.9
Hemicellulose		12.3				10.2	6.6
Xylan	**9.3**		8.0	15.7	12.0		
Arabinan	**1.7**		2.5	3.6	2.5		
Galactan	**2.4**						
Manann	**2.2**						
Klason lignin	**37.0**	19.3	17.0	29.4	32.1	25.3	15.6
Soluble lignin	**8.0**	1.7	1.8	2.9			
Protein	**4.6**		5.7	7.3			9.2
Ash	**5.7**	4.8	4.3	4.2	2.9		3.1
Others (by difference)	**18.5**			7.4		9.5	
Reference	**Present study**	Alves-Ferreira et al. (2019b)	Alves-Ferreira et al. (2017)	Alves-Ferreira et al. (2019a)	Fernandes et al. (2018)	Carrión-Prieto et al. (2017)	Ferro et al. (2015)

ESR(Sx): Soxhlet extract of ESR with different solvents; RM(Sx): Soxhlet extract of RM with different solvents; RM: Raw material without any previous treatment.

may justify the difference observed in lignin content. Also, in addition to the differences in the starting material (extracted by different procedures versus raw), the place and time of harvest and in particular the age of the plant are also factors that can influence the lignocellulosic composition of this species, thus justifying the observed differences. Nevertheless, the high content in lignin of the ESRs for both *C ladanifer* and *C. lusitanica* species here revealed suggests an additional potential for its valorisation.

3.2. Total phenolics, flavonoids and tannins content

The yield of the UAE phenolic-rich extracts obtained from the ESRs of *C. lusitanica* and *C. ladanifer*, and also the polyphenolic content for both UAE extracts and DCWs for both biomass species, are detailed in Table 3. The results show that the yield of phenolic-rich extract for *C. ladanifer* was ca. 3 times higher than the yield obtained for *C. lusitanica*, independently of the extraction solvent, which indicates that this biomass is potentially richer in these compounds. The total phenolics, tannins and flavonoids content obtained show again that *C. ladanifer* extracts are richer in these compounds than *C. lusitanica* extracts, which are in agreement with the yields obtained and the phenolic profiles shown below (Fig. 3).

The 70 % acetone extracts for both species presented higher total phenolics, flavonoids and tannins content than the ethanol extracts. The DCW of *C. lusitanica* and *C. ladanifer* also showed the presence of phenolic compounds, mostly tannins. Regarding *C. lusitanica*, the total phenolic content for DCW was lower than UAE extracts, while for *C. ladanifer*, the total phenolic content of DCW was superior than UAE extracts with ethanol, but lower than UAE extracts with 70 % acetone. Overall, it was found that extraction with 70 % acetone was more efficient in removing phenolic compounds than the other solvents used for both species.

Table 3
Extraction yield, total phenolics, flavonoids and tannins of distiller condensation waters and the different extracts obtained from *Cupressus lusitanica* and *Cistus ladanifer* distillation by-products.

	Cupressus lusitanica			*Cistus ladanifer*		
	DCW	ESR (EtOH)	ESR(70 %Ace)	DCW	ESR (EtOH)	ESR(70 %Ace)
Extraction yield (%)		4.02	3.85		13.3	11.0
Total Phenolics (mg GAE/g extract)	139.7 ± 0.2	178.9 ± 0.4	251.3 ± 0.6	209.6 ± 0.2	177.5 ± 0.2	275.6 ± 0.0
Flavonoids (mg QE/g extract)	1.3 ± 0.1	4.5 ± 0.1	6.3 ± 0.0	11.5 ± 0.8	12.3 ± 0.2	15.2 ± 0.1
Tannins (mg GAE/g extract)	86.8 ± 0.3	23.0 ± 0.2	82.2 ± 0.4	133.3 ± 1.2	110.5 ± 0.6	116.6 ± 0.8

DCW: Distiller condensation water; ESR: Extracted solid residue; GAE: Gallic acid equivalents; QE: Quercetin equivalents.

Although it is known that *Cupressus* trees are rich in phenolics, flavonoids and tannins (Harraz et al., 2018), there are very few studies about their composition in *C. lusitanica*. Guimarães et al. (2010) were the first to disclose the phenolic estimation and antioxidant activity of a 50 % methanolic extract from *C. lusitanica* fresh leaves harvested in North-eastern Portugal, revealing values of 30 mg GAE / g extract. The reported value was much lower than that obtained in the present study, probably due to the different material used (fresh leaves against dried aerial parts) and also different extraction solvents and conditions. No references were found in literature for the flavonoids and tannins content of *C. lusitanica*. The only results found for *Cupressus* genus was the study of Selim et al. (2014), which determined the total flavonoid content of a methanol extract from *Cupressus sempervirens* aerial parts (leaves) as being 53 mg QE / g of sample, which is approximately ten times higher than the values here reported for *C. lusitanica*. However, some data could be found for other Cupressaceae species, particularly for *Juniperus* species. A study of water and 80 % ethanol extracts of chopped dried leaves from five *Juniperus* species disclosed a total phenol content ranging from 4.0 to 139 mg GAE / g for the water extract and 111–206 mg GAE / g for the 80 % ethanol extract (Orhan et al., 2011). The same study reported the flavonoid content in quercetin equivalents (mg QE / g extract). Even though results are within a wide range according with the different *Juniperus* species, results from the present study are within the same order of magnitude with the ones reported by Orhan et al. (2011) for total phenols of *C. lusitanica* extracts obtained with the same solvent system. However, flavonoid content for the 80 % ethanolic extracts were generally more than ten times higher than the ones of water extracts for *Juniperus* species, while our results present flavonoid content for *C. lusitanica* of 5–6 times higher for ethanol and 70 % acetone extract than for water extract, respectively.

Regarding *C. ladanifer*, the previous study by Alves-Ferreira et al. (2019b), obtained values between 271−286 mg GAE/g extract for ESR (50%EtOH) by UAE at 50 °C for 60 min. These values are higher than the ones here obtained with pure ethanol, but within the range of the ones obtained with 70 % acetone extracts. In the same study, flavonoids

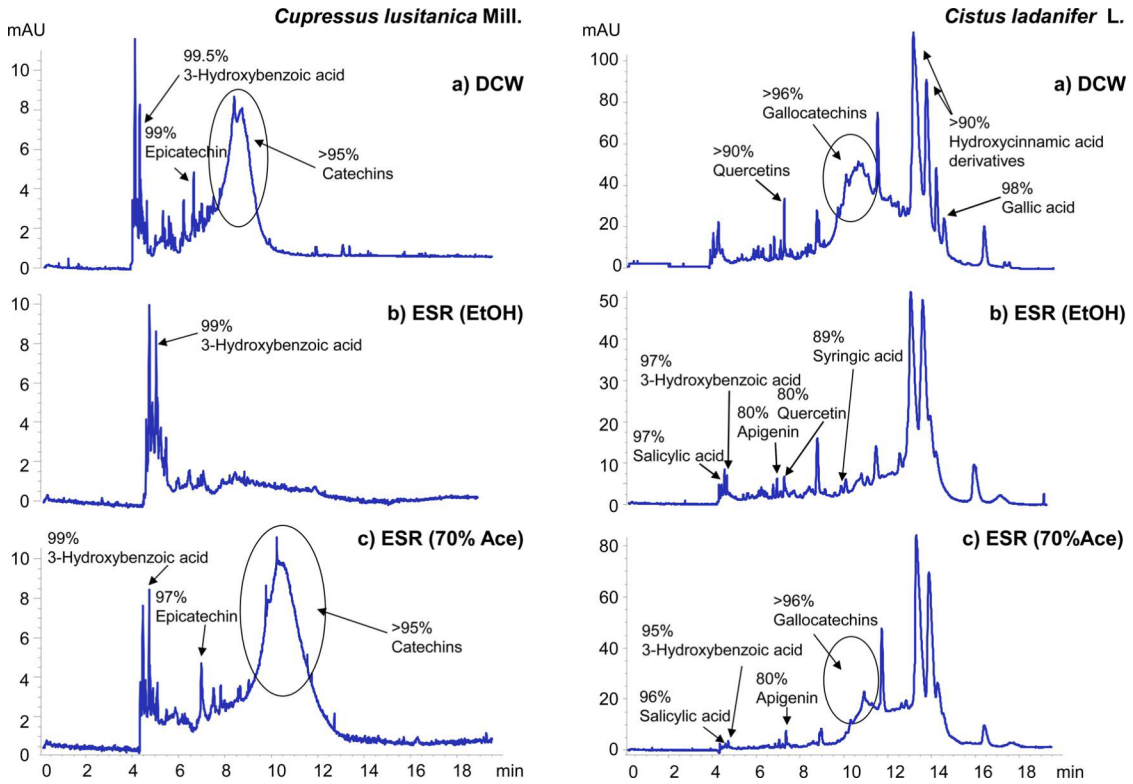

Fig. 3. Electropherograms at 280 nm showing the phenolic profiles obtained for obtained for *C. lusitanica* and *C. ladanifer* wastes distilled by-products: a) distiller condensation waters (DCWs), and ultrasound-assisted extracts from the extracted solid residues obtained with b) EtOH (ESR(EtOH) and c) 70 % acetone (70 % Ace). Matching % was obtained by comparison with authentic standards. See text for CZE conditions.

(33−39 mg CE/g extract) and tannins (22−26 mg CE/g extract) were determined. The flavonoids content were higher than the ones in the present study, but tannin values were lower. These latter results complement the ones here disclosed and show the effect of different conditions of extraction (higher temperature and time) and also of solvent used (50 % ethanol allows the extraction of water soluble glycosides and sugars, which are also accounted in the Folin method).

Flavonoids and tannins were determined using a different procedure than in the present study, also justifying these differences. Dudonné et al. (2009) found values of 103 mg GAE/g extract of total phenolics for aqueous extracts of initial plant material harvest in Spain, which were lower than the results shown in Table 3, independently of the extraction solvent. The differences observed may also be due to the origin and conditions of the material used. Also, total phenolics and flavonoids content of ethanolic (95 %) and acetone/water (60:40) extracts obtained by reflux of wood/stalks, bark and leaves of *C. ladanifer* was determined by Andrade et al. (2009), disclosing values of 255 and 335 mg GAE/g extract, respectively, and 21 and 23 mg QE/g extract, respectively. These authors also concluded that acetone extracts give rise to more compounds than ethanol. Values were higher comparing with the present study for ethanolic and acetone/water extracts, which is expected considering that they also include results for bark. Again, these variations are also dependent of several factors, including the state of the material, harvesting time and area, and the extraction conditions. Nevertheless, *C. ladanifer* extracts here studied showed to be rich in phenolics, supporting the additional potential for valorisation of these residues as a source of bioactive compounds.

3.2.1. *Phenolic profile by Capillary Zone Electrophoresis*

The phenolic profile from the DCWs and the different UAEs extracts obtained from *C. lusitanica* and *C. ladanifer* distillation by-products are shown in Fig. 3. Both species extracts present very rich and complex, nevertheless different phenolic profiles. Each species shows a characteristic profile, similar for all the extracts within each species, except for the extracted solid residue ethanolic extract of *C. lusitanica*. It is noteworthy from the e-grams in Fig. 3 that *Cistus* extracts present a higher and more complex phenolic content than *Cupressus* extracts. Phenolic compounds identification and the % matching with the available authentic standards are also shown in the e-grams. Catechins were the major compounds found for both species extracts and also 3-hydroxybenzoic acid was found as a common compound. Gallocatechins, hydroxycinnamic acid derivatives and gallic acid were only identified in *Cistus* extracts. As previously mentioned, results for the phenolic composition of *Cupressus* sp. are scarce, particularly for *C. lusitanica*. Cowan et al. (2001) reported two lignans (arctigenin and matairesinol) in a dichloromethane extract of stems from *C. lusitanica* and referred also the presence of tropolones in this species. Romani et al. (2002) reported a method of high performance liquid chromatography with diode-array detector combined with mass spectrometry (HPLC-DAD-MS), for the identification and quantification of flavonoids and bioflavonoids present in 70 % hydroalcoholic extracts of Cupressaceae green leaves, including *C. lusitanica*. In their study, Romani et al. (2002) identified quercetin-glucoside and quercetin-rhamnoside, cupressusflavone, amentoflavone, robustaflavone and methylamentoflavone in *C. lusitanica* fresh leaves.

The current study refers to by-products and extracts from a steam distillation procedure of 1 h:30 min (Tavares et al., 2020), which have suffered hydrolysis and thermal degradation in the different preparation steps. Therefore, it was only expected to find non-conjugated forms of the flavonoids and phenolics. Nevertheless, only 3-hydroxybenzoic acid and catechins, could be identified with matching confidence ($\geq$ 95 %), namely epicatechin. Catechin and epicatechin are the building blocks of proanthocyanidins, a type of condensed tannins, which justifies the values found for the tannins content in *C. lusitanica* DCW and ESR (70 % Ace) extracts.

Opposite to *C. lusitanica*, *C. ladanifer* phenolic composition is well

known and studied, as this is an important aromatic plant widely used in the perfumery industry. Phenolic compounds identified in *C. ladanifer* steam-distillation by-products, with the available standards, (Fig. 3), are in agreement with literature data, although these compounds content in *Cistus* species are highly variable (Papaefthimiou et al., 2014). Our results revealed high concentrations of tannins, which have also been determined in various *Cistus* species, including *C. ladanifer*, such as gallic acid, as reported by Barrajón-Catalán et al. (2011). Several flavonoids belonging to the groups of flavones, flavonols and flavon-3-ols, such as apigenin, quercetin, gallic acid and gallocatechins here identified, were previously detected in *C. ladanifer* (Chaves et al.,1997; Fernández-Arroyo et al., 2010; Barrajón-Catalán et al., 2011) and also in the Soxhlet ethanolic extract of *C. ladanifer* analysed by Alves-Ferreira et al. (2019b). Although hydroxycinnamic acid derivatives in *C. ladanifer* were reported before (Chaves et al., 2001; Herranz et al., 2006), they were not present in the Soxhlet ethanolic extract analyzed by Alves-Ferreira et al. (2019b), but were here identified with a matching > 90 %.

3.3. Antioxidant activity

The antioxidant potential of UAE extracts and DCW of *C. lusitanica* and *C. ladanifer* distillation by-products are summarized in Table 4. In general, extracts of *C. ladanifer* showed higher antioxidant activity than those of *C. lusitanica*. Also, UAE with 70 % acetone were the ones with highest results determined by all methods for both species, except for superoxide, which was higher for DCWs. The results here obtained show a valid assessment for the potential use of DCWs and ESRs extracts as natural antioxidants in different industries, such as food, cosmetic and pharmaceutical. The potential antioxidant activity of these extracts was previously evaluated, and their results expressed in terms of % inhibition (Tavares et al., 2019) and the same tendency was observed. Antioxidant activity is usually associated with the presence of phenolic compounds (Ballesteros et al., 2017). The activity observed in the present study may be due to the type of phenolic compounds, namely catechins and gallocatechins, as well as other flavonols and flavanols. Only one study in literature refers the antioxidant activity of *C. lusitanica* extracts (Guimarães et al., 2010) suggesting a maximum of 80 % DPPH inhibition for this species methanolic extracts.

Cistus species rich in phenolic compounds, especially flavonoids, are known to demonstrate significant antioxidant activity (Papaefthimiou et al., 2014), namely, aqueous extracts were able to generate strong antioxidant activities in a dose-dependent manner, using several free radical scavenging methods. Water and ethanol extracts of this species aerial parts showed high scavenging ability of DPPH radical, the water extracts showing 70–95 % inhibition and 95 % in case of the ethanolic extracts (Zidane et al., 2013). Guimarães et al. (2010) also obtained a maximum inhibition of 90 % with the leaf methanolic extracts, using the DPPH method.

The results here obtained for the antioxidant activity support the fact that aerial parts of cypress trees (Harraz et al., 2018; Ibrahim et al., 2009; Kuiate et al., 2006) including *C. lusitanica* (Romani et al., 2002; Selim et al., 2014) and also species of Cistaceae, including *C. ladanifer* (Barros et al., 2013; Attaguile et al., 2000) have been traditionally used as remedies in folk medicine to treat several diseases, including anxiety, headaches, asthma, infection, inflammation, diabetes and various types of cancer, among others. These effects are mainly attributed to the bioactivities of essential oils and secondary metabolites such as phenolics present in these species, in particular to their antioxidant and anti-inflammatory activity (Alam et al., 2016). The same bioactive compounds are also present in these species biomasses. It is important to refer that hydroxycinnamic acid derivatives were identified in DCW and ESR extracts of *C. ladanifer*, and salicylic acid was found in ESR (70 % Ace), partly justifying the anti-inflammatory effect referred for this species (Barros et al., 2013). In fact, recently, the anti-inflammatory activity of steam-distillation hydrolates and by-products from both *C. lusitanica* and *C. ladanifer* biomasses were evaluated for the first time, with *C. ladanifer* evidencing higher activity for all the samples tested (Tavares et al., 2019 and 2020).

Table 4
Antioxidant activity of distiller condensation waters and the different extracts obtained from *Cupressus lusitanica* and *Cistus ladanifer* distillation by-products.

	Cupressus lusitanica			*Cistus ladanifer*		
Antioxidant activities	DCW	ESR (EtOH)	ESR(70 %Ace)	DCW	ESR (EtOH)	ESR(70 %Ace)
ABTS TEAC (mmol/g extract)	1.6 ± 0.0	0.8 ± 0.2	2.0 ± 0.1	2.6 ± 0.0	1.8 ± 0.1	4.1 ± 0.0
Superoxide AAE (mg/g extract)	768.4 ± 2.7	724.5 ± 2.4	753.2 ± 2.2	813.0 ± 6.2	703.8 ± 1.3	795.0 ± 2.8
Xanthine AE (mg/g extract)	85.5 ± 0.2	70.6 ± 0.2	73.5 ± 0.2	87.6 ± 0.7	71.4 ± 0.1	76.4 ± 0.3
Chelating EDTAE (mg/g extract)	16.7 ± 0.1	14.7 ± 0.7	16.3 ± 0.1	15.1 ± 0.2	13.8 ± 0.0	15.8 ± 0.1

DCW: Distiller condensation water; ESR: Extracted solid residue; TEAC: Trolox equivalent antioxidant capacity; AAE: Ascorbic acid equivalent; AE: Allopurinol equivalent; EDTAE: EDTA equivalent.

DCWs and ESRs extracts are thus an abundant source of natural phenolic compounds, tannins in particular, that can be exploited as building blocks in different industries (Rahim et al., 2019), not only as natural antioxidants, dyes and food additives, but also as raw materials for producing medical drugs or other chemicals such as polymers (Li et al., 2019; Cavaca and Afonso, 2018; Duval and Avérous, 2016; Garcia et al., 2016; Ramires et al., 2010).

4. Conclusions

Distiller condensation waters and extracted solid residues resulting from steam-distillation of essential oil from wastes of *Cupressus lusitanica* and *Cistus ladanifer*, were here characterized and their phenolic composition as bioactive compounds was evaluated. The ethanol and 70 % acetone extracts obtained from these residues and the distiller condensation waters were here studied for the first time and have remarkable phenolic content and antioxidant activity, the extent of which depending on the solvent used and the original species. Results indicate some correlation of the antioxidant activity with the type of phenolic compounds identified, namely catechins and hydroxycinnamic acid derivatives, and other flavonoids such as quercetins. Thus, the present work together with previous researches shows that forest biomass has a great potential as a renewable resource for recovery of bioproducts, in particular phenolic compounds, to be used as building blocks in different industries. Within a biorefinery context and towards zero waste, the use of the remaining extracted solids for pellets production, alone or together with other biomasses, is currently an ongoing study.

Credit author statement

Cláudia S. Tavares, A. Cristina Figueiredo and Luísa B. Roseiro: conceived and designed the research, conducted the experiments and wrote the manuscript. Alice Martins, M. Graça Miguel, Florbela Carvalheiro, Luis C. Duarte and José Gameiro: conducted some parts of the experiments. A.Cristina Figueiredo and Luísa B. Roseiro: supervised the work. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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