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Anthocyanins, Phenolic Compounds, and Antioxidants from Extractions of Six *Eucalyptus* Species

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Abstract: The leaves of *Eucalyptus* have multiple biological activities such as antimicrobial, antiseptic, antioxidant, and antifungal. A Soxhlet extraction, SLE, and HD were used to obtain extracts from the leaves of six *Eucalyptus* species, *E. globulus*, *E. oblicua*, *E. pavaflora*, *E. camaldulensis*, *E. viminalis*, and *E. nitens*, and to study their antioxidant capacity. Solvents such as acetone, dichloromethane, ethanol, hexane, methanol, and water were used to study how polarity influences extraction yields. The SLE method achieved higher or similar yields, depending on the species and its composition, than the Soxlet method at a temperature of 50 °C. The highest yields were obtained with *E. viminalis* with methanol (42.5 wt.%), the highest phenolic content was obtained with *E. nitens* with methanol (124.17 mg GAE/g of extract), and the highest anthocyanin levels obtained were with *E. nitens* with hexane (5.05 mg CC/g of extract). *E. nitens* obtained almost five times more phenolic content than *E. globulus*; therefore, it is the most promising species. The high content of the compounds analysed confirm the good potential of these species to obtain value-added compounds. Our results demonstrate that the differences in the extract contents depend on the polarity of the solvents used. In addition, the use of these species will reduce the residue in the forest, which is greatly beneficial.

Keywords: *Eucalyptus*; Soxhlet extraction; solvents; gallic acid; anthocyanins

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1. Introduction

Eucalyptus is a genus of over 900 species and subspecies of flowering trees and shrubs found in the Myrtaceae family [1]. Originally from Australia, nowadays *Eucalyptus* can be found all over the world [2], and with 18 million ha planted in over 90 countries, it is one of the most widely scattered hardwoods worldwide [3]. *Eucalyptus* spp. are fast growing plants with the capacity to tolerate harsh environments such as wild fires, drought, and soil acidity [4]. Because of this, numerous species of *Eucalyptus* are used in the wood industry for the production of pulp, timber, and paper [5], while the bark, leaves, and branches are usually considered by-products utilised for energy production [6,7]. Even the production of pellets using mixtures of microalgae and *Eucalyptus* has been studied, reducing the negative impact on ecosystems and reducing waste. In Galicia, in north-western Spain, the first specimen planted was *Eucalyptus globulus*, in 1850. Nowadays, the numbers of species of genus *Eucalyptus* found in Galicia include 40 taxa (37 species and 3 subspecies), including several hybrids [8]. A study by Picos [9] showed that in Galicia, in 2017, 8.5 million of m^3 of wood had been cut, which represented 47% of the total wood cut in Spain, and accounted for 1.9% of the round-wood production in the European Union. Fifty per cent of the total wood cut was eucalyptus. The forest industry in Galicia employed 20,320 people in 2017 and had a turnover of 2200 million euros [9,10].

In addition to its use to obtain paper pulp, other uses have been analysed that highlight its potential in other industries. The medicinal properties of *Eucalyptus* spp. have been reported in various studies [2] and high amounts of polyphenols are known to be found in *Eucalyptus* [4,11]. Polyphenols are complex bioactive molecules found naturally in trees and plant-based foods such as berries, cocoa, tea, and coffee [12,13]. Polyphenols can be divided into flavonoids, phenolic acids, stilbenes, and lignans [14]. Research on the characteristics and properties of polyphenols have linked these molecules to the prevention of cancer, diabetes, osteoporosis, coronary disease, neurodegenerative diseases, and diabetes mellitus [15].

Eucalyptus leaves are being widely studied as they contain volatile essential oil, with multiple widespread biological activities such as antimicrobial, antiseptic, antioxidant, and antifungal [16,17]. The leaves are evergreen, simple, hairless, and with a smooth margin, in other words without teeth or serrations. They usually have two types of leaves: those of the young specimens are wide and often lack stalks, while those of the adult specimens are more elongated, alternate, and always have an obvious stalk. The leaves can be classified according to the venation and the shape. The adult sheet has a central nerve and pinnate sides that are usually welded on a line next to the margin. According to the venation, they can be classified into almost parallel, oblique, and transverse, and according to shape, they can be classified into lanceolate, broadly lanceolate, narrowly lanceolate, elliptic, ovate, linear, and oblique [8].

Eucalyptus leaves are a great source of antioxidants, particularly flavonoids, which offer protection against from oxidative stress and free radical damage. The flavonoid subgroups are flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins, and chalcones [18]. The main flavonoids presented in eucalyptus are catechins, isorhamnetin, luteolin, kaempferol, phloretin, and quercetin [19]. On the other hand, *Eucalyptus* leaf extract can also be used to impart new functionality to textile fibres, such as the production of anti-UV and antimicrobial cotton fabrics through dyeing, thus improving cotton material [20]. This application works to mitigate the appearance of cyanotoxins in the water, toxic metabolites [21], ensuring the optimal state of water security. Other applications where *Eucalyptus* leaves extract can be used are in the mild steel industry as a source for inhibiting corrosion in acidic media [22], and as tool to control and reduce the proliferation of cyanobacteria in aquatic environments [23]. Moreover, there has been an increasing interest in the use of *Eucalyptus* leaves extracts from different fields such as pharmaceutical [11,24], cosmetic [25], and food industries [11,24].

The aim of this study was to determinate the most efficient methodology for obtaining extracts from the leaves of six *Eucalyptus* species, *E. globulus*, *E. oblicua*, *E. pavaflora*, *E. camaldulensis*, *E. viminalis,* and *E. nitens*. For this purpose, three extraction methods have been studied (Figure 1): solid-liquid extraction (SLE), which implies a more rational use of solvent quantities, temperature, and extraction time; hydrodistillation (HD), as the most economical method; and the Soxhlet method (Figure 1). However, optimal extraction will depend largely on the polarity of the solvent used, the chemical composition of the compounds to be extracted, the quantity and position of their hydroxyl groups, solvent concentration, temperature, time of contact, particle size, and mass-solvent ratio [26–28]. In this work, six solvents with different polarity were used: water, acetone, ethanol, methanol, hexane, and dichloromethane. The influence of the solvent polarity on the extraction yield was analysed and the extracts obtained were characterised according to their content in total phenols and anthocyanins. Due to the importance of phenolic and flavonoids compounds, studying the viability of the use and exploitation of these species, which are easy to grow and widely spread in the study area, is presented as a promising analysis. In addition to obtaining value-added compounds, we would reduce waste in the forest. The development of this technology promotes land conservation and restoration, as well as innovative industrial development. These goals are part of the EU's political agenda, supported by the green agenda, and included in the United Nations Sustainable Development Goals (SDGs) [26].

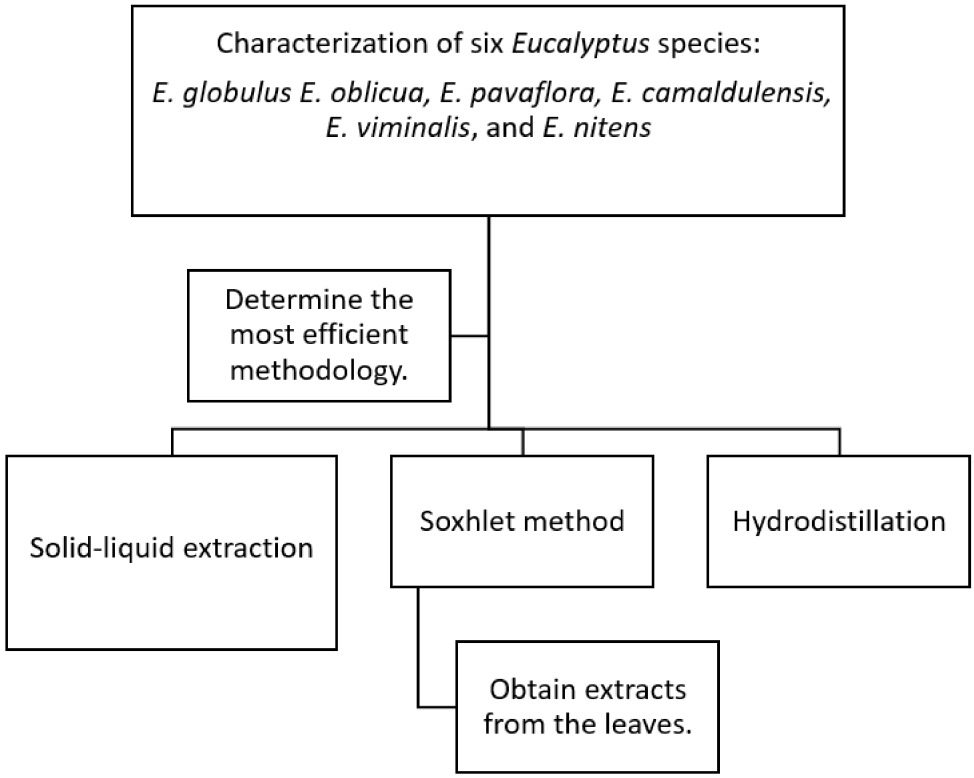

Figure 1. Flowchart of the proposed methodology.

2. Materials and Methods

2.1. Sample Preparation

The eucalyptus leaves were supplied by Lourizán Forestry Research Centre in Pontevedra (Galicia), in north-western Spain. The fresh leaves were washed with distilled water to remove all impurities and then kept in the oven at 50 °C for two days to dry them. The leaves presented a moisture content of 5 wt.%. They were cut into small pieces of around 1 cm. The characteristics of the leaves are shown in Table 1.

Table 1. Leaf size of the six species studied and weights of the samples.

Species	Length (±0.1 cm)	Width (±0.1 cm)	Samples Weight (±0.1 g)
E. camaldulensis	5.0–15.0	0.7–1.5	1.5
E. globulus	12.0–25.0	1.7–3.0	4.0
E. nitens	20–31.0	2.0–3.5	4.0
E. oblicua	10.0–17.0	2.0–5.0	3.0
E. pavaflora	10.0–21.0	2.0–4.0	4.0
E. viminalis	8.0–17.0	0.9–2.4	4.0

2.2. Soxhlet Extraction

Studies have shown that Soxhlet extraction is the most effective method for the extraction of phenolic compounds [6,29–31]. All the solvents used in this study were purchased from Fisher and Sigma Aldrich. They all had a 99–100% purity. Their characteristics are shown in Table 2. The select solvents were water (W), acetone (AC), ethanol (ETH), methanol (MTH), hexane (HEX), and dichloromethane (DCH).

Table 2. Characteristics of the solvents used.

Solvent	Type of Solvent	Dielectric Constant F/m	Polarity			Boiling Temperature (°C)	Density (g/mL)
			High	Medium	Low		
Acetone (CH_3COCH_3)	dipolar aprotic	20.7		X		56	0.786 (25 °C)
Dichloromethane CH_2Cl_2	Non-polar	9.1			X	40	1.326 (20 °C)
Ethanol (CH_3CH_2OH)	polar protic	24.3	X			79	0.789 (20 °C)
Hexane ($CH_3CH_2CH_2CH_2CH_2CH_3$)	non-polar	2.02			X	69	0.655 (25 °C)
Methanol (CH_3OH)	polar protic	33	X			65	0.791 (20 °C)
Water (H_2O)	polar protic	80	X			100	1.000 (20 °C)

Every sample was mixed with 200 mL of each solvent. The mixture was introduced for six hours into a Soxhlet extractor, in which six extraction cycles took place. After extraction, the samples were introduced in a IKA RV 3V rotary evaporator (IKA, Staufen, Germany) for solvent removal at 100 °C for 24 h, and then weighed. All extracts were placed at room temperature before determining the extraction yield. Three experiments were carried out dividing every sample in smaller batches. The results were calculated by arithmetic mean.

Total extraction yield was calculated by Equation (1):

$$Y_{total} = \frac{m_{extract}}{m_{raw}} \times 100 \quad (1)$$

where:

$m_{extract}$ is the mass of extract weighted after evaporation solvent

m_{raw} is the dry mass of the leaves.

2.3. Batch Solid-Liquid Extractions (SLE)

The SLE assays were carried out with the above compounds in Soxhlet extractions. Leaves of each species were extracted into a filter bag with 100 mL of the sample solvent for 7 h by shaking at 300 rpm. The solvent was kept under its own boiling point, and the yield was studied at 20, 35, and 50 °C. The liquid solutions were then evaporated to dryness at a rotary evaporator, followed by redissolving the extract in a pre-weighed sample holder, and final drying was carried out in an oven to verify the constant mass of the extract. The total extraction yield was calculated using Equation (1).

2.4. Hydrodistillation (HD)

The HD tests were performed with leaf samples (approx. 90 g), ground (particle size less than 2 mm) and unground, to evaluate the effect of particle size on extraction yield, and with deionised water (approx. 800 mL) for 3 h in a Clevenger-type apparatus. The hydrodistillation product corresponds to the fraction of extractives that was vaporised, condensed, and collected in a side collection vessel.

2.5. Chemical Characterisation

Based on the best performing extraction method, the following analyses were performed:

The total phenol content of extracts was determined by the Folin-Ciocalteu method [32,33]. An Agilent spectrophotometer, the Cary-60 VIS-UV (Agilent Technologies, Santa Clara, CA, USA), with double internal beam and pulsing xenon lamp was used, and absorbance was read at 760 nm. A gallic calibration curve at five gallic acid concentrations (0, 4, 10, 25, 50 mg/L) was obtained. The procedure described for the standard gallic acid was followed and the absorbance was recorded for each extract. Samples were prepared in triplicate for each analysis and the average absorbance value was used to plot the calibration curve to determine the level of phenols in the extracts. The total phenolic content of the extracts

was expressed as mg gallic acid equivalents (GAE) per gram of sample on a dry weight basis (mg/g).

Anthocyanins measurements were done by the acid hydrolysis in butanol method [34]. The absorbance was recorder at 530 nm, absorption maximum observed to anthocyanin [30], and the results were expressed as based on cyanine equivalent. A cyanine calibration curve at five concentrations (0, 5, 10, 15, 30 mg/L) was obtained. A standard calibration method was followed, the commercial cyanine chloride standard, 1 mg, was dissolved in 1 mL of methanol and the 5 mL flask was made up to the mark with distilled water. From this solution, which is 200 ppm, the necessary dilutions are prepared to make the standard line. The results were expressed as mg cyanine chloride /g extract.

Antioxidant activity was assessed by two methodologies: (1) ABTS· + method; 3 mL of ABTS· + solution was mixed with 10 µL of extracts. A decrease in absorbance was measured at λ = 734 nm [30]; (2) CUPRAC assay: the CUPRAC solution included copper (II) chloride (0.01 M in water), ammonium acetate buffer (0.001 M, pH = 7), and neocuproine (0.0075 M in ethanol) (ratio 1:1:1:1). Then, 3 mL of CUPRAC reagent was mixed with 10 µL of extracts. An increase in absorbance was recorded at λ = 450 nm [31].

2.6. Statistical Analysis

The values obtained were entered into statistical analysis using statistical Package for the Social Sciences (SPSS) 16.0 version (SPSS, Inc., Headquarters, Chicago, IL, USA). All experiments were conducted in triplicates. The average of multiple measurements (triplicates or more) was listed in the tables together with the standard deviations. Comparisons were performed by a Student's test and Kruskal-Wallis test. The statistical results confirm the hypothesis that the differences between the results are either not significant ($p > 0.05$), significant ($0.001 < p < 0.05$), or highly significant ($p < 0.001$).

3. Results

3.1. Total Extraction Yield

The results present in Figure 2 show important differences among solvents. High polarity solvents produced the highest yields (32.3–42.5 wt.% with methanol, 30.8–38.7 wt.% with ethanol, and 22.3–35.2 wt.% with water), followed by solvents with medium polarity (19.3–29.5 wt.% with acetone), and non-polar solvents (9.4–19.6 wt.% with hexane 15–19.5 wt.% with dichloromethane).

Finally, the results presented in Table 3 provide information on the expected productivity when approaching the production of these *Eucalyptus* species with boiling water. Although HD methods provide lower yields, the results are similar to those obtained by SLE and Soxhlet with hexane. Despite the low productivity obtained with hexane, it is one of the most widely used solvents in industrial oil production [32].

3.2. Extract Composition

The levels of total phenolics and anthocyanins obtained varied among species (Tables 4 and 5). Our results show the highest phenolic content was obtained 124.17 mg GAE/g of extract for *E. nitens* with methanol. On the other hand, the lowest phenolic content obtained was 1.57 mg GAE/g of extract for *E. oblicua* with hexane.

Phenolic content was higher for all five species of *Eucalyptus* when using the polar solvents methanol (20.10–124.17 mg GAE/g of extract) and ethanol (17.72–112.57 mg GAE/g of extract), than when using non-polar solvents such as hexane (1.57–8.06 mg GAE/g of extract) (Table 4). The anthocyanin content varied greatly depending on the species and solvent (Table 5).

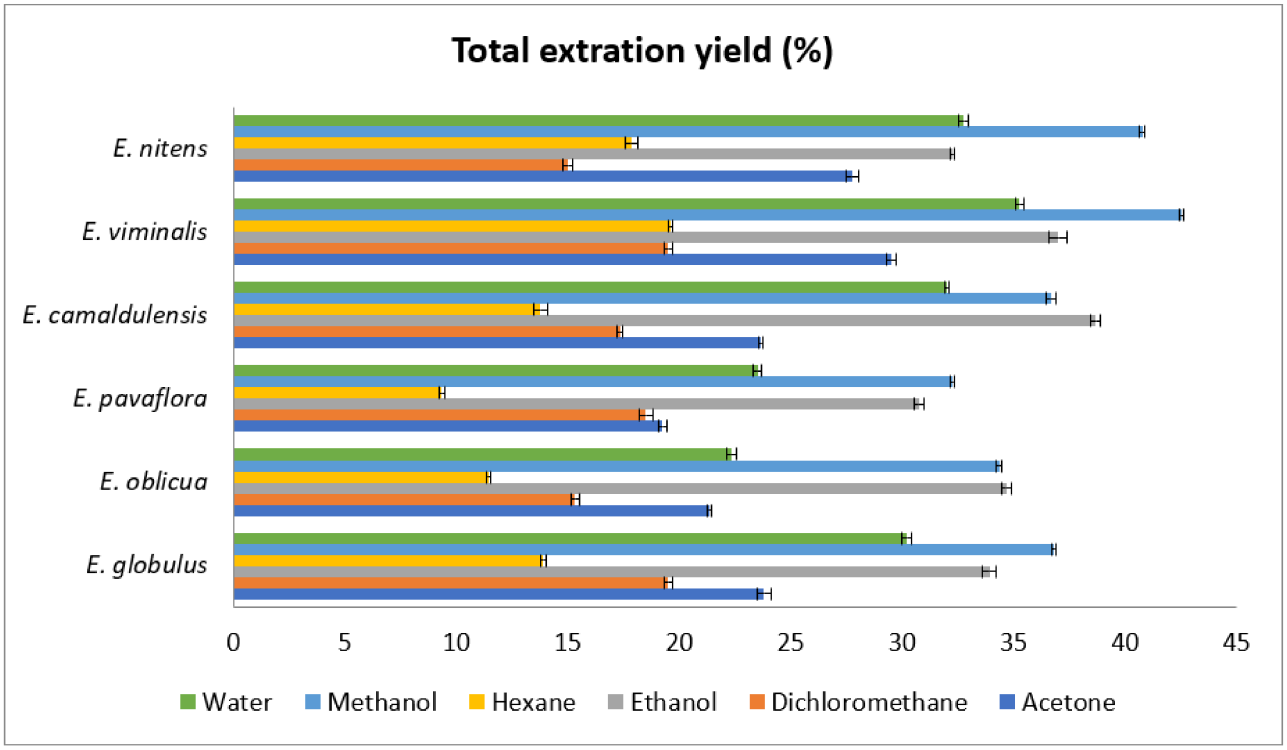

Figure 2. Extraction yields obtained from leaves of six *Eucalyptus* species mixed with solvents of different polarity, using a Soxhlet extractor.

Table 3. Total extraction yield results for SLE and HD methods (%).

Solvent	Method.	*E. globulus*	*E. oblicua*	*E. pavaflora*	*E. camaldulensis*	*E. viminalis*	*E. nitens*
DCH	SLE 20	10.23	8.72	13.44	10.12	15.28	11.10
	SLE 35	15.33	9.21	14.21	11.49	20.62	17.32
MTH	SLE 20	23.11	20.54	19.76	22.14	24.71	23.18
	SLE 35	30.82	23.66	28.22	34.11	36.29	32.17
	SLE 50	36.12	29.74	32.14	40.87	42.11	40.26
ETH	SLE 20	15.17	8.52	20.08	12.44	18.33	14.01
	SLE 35	19.33	9.44	26.76	28.19	29.10	30.45
	SLE 50	32.15	9.78	31.11	35.87	34.52	30.74
HEX	SLE 20	5.77	3.22	6.54	7.08	9.21	6.24
	SLE 35	6.75	4.18	7.26	10.05	14.70	10.83
	SLE 50	11.90	4.97	8.94	13.27	18.43	18.22
AC	SLE 20	11.33	12.07	10.27	14.00	13.57	9.08
	SLE 35	15.80	14.66	11.35	14.35	15.21	10.74
	SLE50	24.51	17.14	15.14	20.17	22.14	19.72
W	SLE 50	20.71	17.21	16.16	14.21	15.14	13.11
	HD 50	12.11	11.03	10.44	13.18	15.21	9.07

Water (W), acetone (AC), ethanol (ETH), methanol (MTH), hexane (HEX), and dichloromethane (DCH).

Table 4. Total phenolics obtained, expressed as mg gallic acid/g extract.

Total Phenolics (mg GAE/g of Extract)						
Solvent	***E. globulus***	***E. oblicua***	***E. pavaflora***	***E. camaldulensis***	***E. viminalis***	***E. nitens***
DCH	$10.09 \pm 0.16^{b,c,d,e,f}$	$7.96 \pm 1.63^{b,c,d,e,f}$	$8.87 \pm 0.49^{b,c,d,e,f}$	$8.52 \pm 0{,}01^{b,c,d,e,f}$	$14.23 \pm 1.00^{b,c,e,f}$	$24.99 \pm .1{,}13^{b,c,d,e,f}$
MTH	$26.88 \pm 2.14^{b,c,d,e,f}$	$20.10 \pm 1.87^{a,d,e,f}$	$20.27 \pm 0.60^{a,d,e,f}$	$49.73 \pm 4.73^{a,c,d,e,f}$	$44.13 \pm 1.70^{a,c,d,e,f}$	$124.17 \pm 6.46^{a,d,e,f}$
ETH	$17.72 \pm 0.78^{a,b,d,e,f}$	$20.82 \pm 0.68^{a,d,e,f}$	$19.81 \pm 0.24^{a,d,e,f}$	$30.84 \pm 1.03^{a,b,d,e,f}$	$29.32 \pm 0.14^{a,b,d,e,f}$	$112.57 \pm 1.75^{a,d,e,f}$
W	$7.68 \pm 0.41^{a,b,c,e,f}$	$4.23 \pm 0.17^{a,b,c,e,f}$	$15.66 \pm 0.15^{a,b,c,e,f}$	$17.32 \pm 1.34^{a,b,c,e}$	$16.95 \pm 0.57^{b,c,e,f}$	$36.21 \pm 7.15^{a,b,c,e,f}$
HEX	$2.00 \pm 0.50^{a,b,c,d,f}$	$1.57 \pm 0.24^{a,b,c,d,f}$	$2.99 \pm 0.26^{a,b,c,d,f}$	$2.67 \pm 0.81^{a,b,c,d,f}$	$8.06 \pm 0.57^{a,b,c,d,f}$	$4.11 \pm 0.68^{a,b,c,d,f}$
AC	$14.00 \pm 0.10^{a,b,c,d,e}$	$9.59 \pm 0.20^{a,b,c,d,e}$	$12.10 \pm 0.10^{a,b,c,d,e}$	$15.83 \pm 0.18^{a,b,c,e}$	$15.63 \pm 0.20^{b,c,e,f}$	$10.95 \pm 0.15^{a,b,c,d,e}$

Statistical significances of phenolics values are shown in superscripts letters: a statistically significant differences versus values of DCH extract; b versus values of MTH extract, c versus values of ETH extract, d versus values of W, e versus values of HEX, and f versus values of AC ($p < 0.05$).

Table 5. Anthocyanins obtained, expressed as mg cyanine chloride /g extract.

Anthocyanins (mg CC/g of Extract)						
Solvent	***E. globulus***	***E. oblicua***	***E. pavaflora***	***E. camaldulensis***	***E. viminalis***	***E. nitens***
DCH	$2.32 \pm 0.16^{d,e,f}$	$1.30 \pm 1.63^{d,f}$	$4.57 \pm 0.05^{b,c,d,f}$	$1.92 \pm 0.24^{d,e,f}$	$0.85 \pm 1.01^{b,c,d,e}$	$2.46 \pm 0.29^{b,c,e,f}$
MTH	$2.14 \pm 0.01^{d,e,f}$	$1.91 \pm 0.06^{d,f}$	$2.15 \pm 0.02^{a,e,f}$	$2.12 \pm 0.11^{d,e,f}$	$4.19 \pm 1.71^{a,c,d,f}$	$4.36 \pm 0.55^{a,d,f}$
ETH	$1.75 \pm 0.00^{d,e,f}$	$1.85 \pm 0.06^{d,f}$	$1.91 \pm 0.04^{a,e,f}$	$1.76 \pm 1.03^{d,e,f}$	$3.00 \pm 0.14^{a,b,d,e,f}$	$4.03 \pm 0.22^{a,d,f}$
W	$0.89 \pm 0.01^{a,b,c}$	$0.41 \pm 0.00^{a,b,c,e}$	$2.09 \pm 0.04^{a,e,f}$	$1.03 \pm 0.01^{a,b,c,e,f}$	$1.34 \pm 0.57^{a,b,c,,e,f}$	$2.75 \pm 0.21^{b,c,e,f}$
HEX	$1.00 \pm 0.01^{a,b,c}$	$1.98 \pm 0.06^{d,f}$	$4.16 \pm 0.01^{b,c,d,f}$	$3.64 \pm 0.11^{a,b,c,d,f}$	$4.48 \pm 0.10^{a,c,d,f}$	$5.05 \pm 0.68^{a,d,f}$
AC	$0.56 \pm 0.10^{a,b,c}$	$0.30 \pm 0.00^{a,b,c,e}$	$0.80 \pm 0.11^{a,b,c,d,e}$	$0.45 \pm 0.11^{a,b,c,d,e}$	$0.82 \pm 0.11^{b,c,d,e}$	$0.15 \pm 0.01^{a,b,c,d,e}$

Statistical significances of anthocyanins values are shown in superscripts letters: a statistically significant differences versus values of DCH extract; b versus values of MTH extract, c versus values of ETH extract, d versus values of W, e versus values of HEX, and f versus values of AC ($p < 0.05$).

Two different antioxidant capacity assays (ABTS, CUPRAC) were evaluated for each of the study species. The ABTS assay was based on the ability of the antioxidants to remove ABTS-+, while CUPRAC measured the ability of the antioxidants to reduce Cu(II) to Cu(I). Knowledge of antioxidant activity could be useful for the standardisation of Eucalyptus raw materials. The highest antioxidant activity by ABTS and CUPRAC methods was for acetone extracts (10.72 and 3.44 mmol g^{-1}, respectively for *Eucalyptus globulus*) (Table 6).

Table 6. Antioxidant capacity of extracts of *Eucalyptus* leaves assessed by ABTS, CUPRAC.

Antioxidant Activity (mmol g^{-1} DW)							
Solvent		***E. globulus***	***E. oblicua***	***E. pavaflora***	***E. camaldulensis***	***E. viminalis***	***E. nitens***
AC	ABTS	10.72 ± 0.32^{b}	6.21 ± 0.27^{b}	$7.54 \pm 0.07^{b,c}$	3.45 ± 0.14^{b}	1.75 ± 0.44^{b}	9.43 ± 0.14^{b}
	CUPRAC	$3.44 \pm 0.21^{b,c}$	$1.25 \pm 0.46^{b,c}$	$1.47 \pm 0.21^{b,c}$	$2.12 \pm 0.15^{b,c}$	1.39 ± 0.24^{b}	$3.14 \pm 0.06^{b,c}$
ETH	ABTS	$1.68 \pm 0.07^{b,c}$	$0.85 \pm 0.08^{a,c}$	$1.71 \pm 0.08^{a,c}$	$0.65 \pm 0.13^{a,b}$	$1.01 \pm 0.14^{a,c}$	$1.23 \pm 0.12^{a,c}$
	CUPRAC	$0.49 \pm 0.08^{a,c}$	$0.43 \pm 0.04^{a,c}$	0.99 ± 0.11^{a}	$0.33 \pm 0.02^{b,c}$	$0.45 \pm 0.16^{a,c}$	0.75 ± 0.04^{a}
MTH	ABTS	9.27 ± 0.07^{b}	5.34 ± 0.16^{b}	$4.23 \pm 0.08^{a,c}$	2.42 ± 0.10^{b}	1.55 ± 0.06^{b}	8.08 ± 0.18^{b}
	CUPRAC	$2.50 \pm 0.10^{a,b}$	$2.31 \pm 0.05^{a,b}$	1.00 ± 0.01^{a}	$0.85 \pm 0.06^{b,c}$	1.47 ± 0.14^{b}	0.45 ± 0.09^{a}

Statistical significances of antioxidant capacities values are shown in superscripts letters: a statistically significant differences versus values of acetone extract; b versus values of ethanol extract, and c versus values of methanol extract ($p < 0.05$).

4. Discussion

4.1. Total Extraction Yield Analysis

Al-Sumri et al. [33] studied the effects of solvent type on palm seed oil and found that polar solvents such as methanol and ethanol gave better total yields than those with lower polarity such as acetone. However, they also suggested particle size could influence percentage of extraction yield. Thus, they determined the diminution in particle size caused an increase of yields. Markom et al. [35] discovered that polar solvents, for instance water

and ethanol, gave better yields than non-polar solvents in a study in which they analysed the effects of solvent type on the extraction yield of bioactive compounds in *Pinus niruri*. In addition, incorporating water to acetone and ethanol did increase the extract yield [35]. On the same line, Muhammad Muhayyidin et al. [36] determined that the polar solvents methanol and water increased percentage yields compared to other non-polar solvents. As in other studies, they also suggested that particle size influenced the effectiveness of the extraction, and the larger the particle, the lower the yields obtained.

Extraction yields are different between species: *E. viminalis* gives the highest yields, ranging from 19.5 to 42.5 wt.%, while *E. pavaflora* gives the lowest yields ranging from 9.4 to 32.3 wt.%. Available literature also indicates variation in extraction yields between different species of *Eucalyptus*. Lima et al. [5] reported Soxhlet extraction yields of 11.3% for *E. viminalis*, 8.6% for *E. globulus*, and 14.8% for *E. camaldulensis*. These extractions were done with a 50% aqueous ethanol solution of the barks, which could confirm the influence of solvents and the parts of the plant used on the percentage of extraction. Rodrigues et al. [6] reported for *E. globulus* a Soxhlet extraction yield variation of 7.32 to 30.34 wt.%, obtained with the use of different polarity solvents. Achmad et al. [37] recorded variations of yields of different eucalyptus species leaves from 1.6 to 3.3%. They suggested differences depended on the species, plant growing location, and age of the leaves.

The yield achieved through SLE at different temperatures is expected to reach that obtained by the Soxhlet method. By heating the solvent, the temperature increases the solubility of the solutes, as well as improving the diffusion rate and mass transfer, decreasing the solvent viscosity and surface tension [38]. From the results obtained, it is possible to highlight the importance of temperature for ethanol, hexane, and acetone, which improves their yields by 57–50%, based on the average between the leaves of each species. The temperature benefit to achieve maximum yield is not related to the boiling point of the solvent. For example, hexane and methanol have close boiling points (68.5 °C and 64.7 °C, respectively), while the yields are disparate [39].

4.2. Extract Composition Analysis

The highest anthocyanin levels obtained were 5.05 mg CC/g of extract for *E. nitens* with hexane, and the lowest was 0.30 mg CC/g of extract for E. oblicua with acetone. These variation differences could indicate that different extract amounts will be obtained depending on the solvents used. This difference in distribution may be due to the different species, or in turn, the anthocyanin concentration may be altered by extraction methods, such as different extraction conditions [40]. Furthermore, Student´s test between anthocyanin content and the different solvents was statistically significant. Regarding the analysis of the anthocyanin content, the values obtained with methanol and ethanol were the highest. This is because methanol and ethanol are solvents with higher polarity, and anthocyanins containing a number of polar bonds, particularly the oxonium group, are more soluble in highly polar solvents [41].

The variations obtained for the phenolic composition are in agreement with other similar studies. Limam et al. [41] determined the chemical composition and antioxidant activity of thirteen species of *Eucalyptus* and found total phenols content varied significantly among species. Lima et al. [5] also reported differences in the composition and antioxidant properties of eleven *Eucalyptus* species with ethanol/water Soxhlet extractions. Most of the available literature data, however, centre on *E. globulus*, and they all reported different levels of total phenolics. For example, Dezsi et al. [42] recorded a high amount of phenolic content (235.87 mg GA/g) which they obtained with ethanol. Dos Santos-Ferreira et al. [43], on the other hand, reported a low amount of phenolic compounds (8.09 mg GA/g) but determined that the most efficient methods for extraction of phenolics for *Eucalyptus globulus* were highly polar solvents. In particular, 70/30 methanol/water extract increased phenolic content by 50% (13 mg GA/g) [43]. Pereira et al. [44] also obtained higher amounts of phenolic compounds when using high polarity solvents (47.16 mg GA/g with methanol and 53.42 mg GA/g with water) than with non-polar solvents (4.07 mg GA/g with

dichloromethane). When using a 70/30 methanol/water extract, phenolic contents obtained increased to 62.10 mg GA/g [45]. The differences between concentrations may be due to a number of factors, including plant species, genetic factors, geographical location, soil type, time of collection, herb preparation, drying, and storage [45]. Dos Santos-Ferreira et al. [43] evaluated four solvents, chloroform, ethanol, methanol, and 70% methanol, for the recovery of phenolic compounds from *E. globulus* leaves, and found that methanol and aqueous methanol were the solvents leading to the highest phenolics yield. More recently, Nasr et al. [46] reported that 70% acetone is the most suitable solvent for the extraction of phenolic compounds from *E. camaldulensis* leaves, while the most prominent antioxidant activity is found in extracts obtained with 95% acetone. In this study, the results obtained show that by using AC, high concentrations are obtained, but lower than with MTH or ETH.

Solvent polarity seems to play an important role in the extraction efficiency of phenols. As this study and others have shown, high polarity solvents such as methanol, ethanol, and water will be more efficient than non-polar ones [47]. Student´s test was calculated between phenolic content and polarity ($p = 0.057$), and nonparametric test, Kruskal-Wallis was calculated ($p = 0.497$). There are no statistically significant differences, and the null hypothesis about the distribution between these variables is accepted. However, there are other factors that should be taken into consideration. Particle size has been shown to influence extraction efficiency: the smallest the size of particle samples, the highest the percentage of yields obtained [34,36,48]. Solvent proportion could also improve polyphenol contents by 50%, as stated by Dos Santos-Ferreira et al. [43], Pereira et al. [44], and Rhazi et al. [49]. A dewaxing pre-treatment of the leaves could also result in higher yields as suggested by Rodrigues et al. [6]. Methanol followed by ethanol obtained the value highest of phenolic content. This result is likely due to the greater polarity of methanol, resulting in the more complete extraction of the highly polar antioxidant and phenolic compounds present in the leave of eucalyptus [40].

González- Burgos et al. [19] point out that the different results obtained may be due to multiple characteristics between each method, as well as to the physicochemical properties of the antioxidants, including different environmental pH, hydrophilic-lipophilic properties, different and uneven distribution of antioxidants between lipophilic and hydrophilic media, and differences in the reaction mechanisms and in the qualitative and quantitative composition of the active antioxidant.

Our results are promising: SLE obtained similar results to Soxhlet but with a decrease in the amount of solvent used. Some future lines of research should focus on trying to increase the performance of the extracts obtained. This could be done by carrying out the analyses with different particle sizes, using different solvent proportions, and looking at the leaf age of the plants. Given the variety of *Eucalyptus* species found in Galicia, and the high amounts of wood from this species cut every year in this region, it is only logical to aim for optimising resources and reducing the amount of waste produced every year, such as the leaves, whose oils have been shown to have many applications not only in medicine but also in other industrial fields. This could bring both economic and environmental benefits to the region.

5. Conclusions

Extracts from the leaves of six *Eucalyptus* species, *E. globulus*, *E. oblicua*, *E. pavaflora*, *E. camaldulensis*, *E. viminalis*, and *E. nitens* were obtained through a Soxhlet extraction using different polarity solvents. The SLE yields of the batch converged to the Soxhlet yield in relation to the temperature increase by SLE. The SLE yields exceeded the Soxhlet results for some species when the temperature reached 50 °C, such as with methanol or ethanol. The highest yields were obtained with *E. viminalis* with methanol (42.5 wt.%), the highest phenolic content was obtained with *E. nitens* with methanol (124.17 mg GAE/g of extract), and the highest anthocyanin levels obtained were with *E. nitens* with hexane (5.05 mg CC/g of extract). The highest antioxidant activity was obtained with *Eucalyptus globulus*, 10.72

and 3.44 mmol g^{-1}, by acetone extraction by ABTS and CUPRAC methods respectively. Results varied between species and our study shows that the highest yields and phenolic contents were obtained when using high polarity solvents such as ethanol and methanol. Of the six species studied, *E. nitens* obtained the best results for the compounds analysed. These variations could be due not only to the solvents used but to other factors such as the plant growing location and the age of the leaves. These species are a good source of phenolic and anthocyanin content; their exploitation as an approach to reduce waste would benefit the region, economically and environmentally.

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