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Extraction of flavonoid compounds from bark using sustainable deep eutectic solvents

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ABSTRACT

The use of green solvents in extraction processes, especially for applications of lignocellulosic biomass, has been extensively studied over the last years. Among the range of different green solvents, deep eutectic solvents (DES) show promising results for extraction processes. Therefore, the aim of this work was the use of DES as additives in aqueous mixtures for the selective extraction of flavonoid compounds from the bark of *Larix decidua*. For this purpose, bark has been treated using different solvent ratios consisting of a DES/H_2O mixture (0, 25, 50 and 75 wt%). Two DES were studied, choline chloride:urea and choline chloride:1,4-butanediol. In order to study the success of the extractions, the extracts and the remaining solid fraction were characterised. From the results, it was concluded that the choline chloride:1,4-butanediol (75 wt%) gave the best results, obtaining the richest extracts in flavonoids (383 mg CE/g dried bark extract), as well as those with the highest antioxidant capacity. These good results confirm the capacity of this DES to obtain active biomolecules for further application.

1. Introduction

Biorefineries, defined by the International Energy Agency (IEA) as "the sustainable transformation of biomass into a spectrum of marketable bio-based products (chemicals, materials) and bioenergy (biofuels, power, heat)" (Rombaut et al., 2014), are seen as a key pillar for the development of a bioeconomy-based society. In this way, the wide range of biofuels and bioproducts that can be obtained from a wide variety of biomass sources, especially lignocellulosic biomass, opens up the possibility of developing new, more sustainable processes and products to drive the transformation from the current situation, which is mainly based on petroleum and its derivatives, to one based on the circular bioeconomy. Therefore, all fractions of lignocellulosic biomass must be studied to be valorised, from the structural compounds (cellulose, lignin and hemicelluloses) to the minor compounds such as extractives and inorganic compounds, among others.

Tree barks are an important source of phenolic compounds (Skrypnik et al., 2019). Nevertheless, the extraction and separation of the different compounds in lignocellulosic biomass is not an easy task, mainly because of its structural complexity (Miranda et al., 2012). As a result, the selection of a successful extraction process becomes very important.

Although it is true that tree bark is rich in extractives, especially in comparison with wood, this value normally does not exceed 30% in weight of the bark (Sillero et al., 2019). Moreover, this fraction is composed of a large variety of different compounds (Dou et al.,

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2016), which means that the proportion of each compound is small. For instance, low percentages of phenolic compounds were quantified by Sillero et al. (2019), who reported percentages ranging from 2 to 9% of phenolic compounds in the extracts. Therefore, in order to make the extraction process efficient, it is necessary to choose not only the appropriate extraction technique, but also the most selective solvent. The use of a selective solvent can improve the efficiency of the extraction, reducing the subsequent purification stages. At this point, the principles of green chemistry must also be considered with the aim of carrying out more environmentally friendly processes that eliminate the use of hazardous substances. For these purposes, the most commonly used solvents should be replaced by more environmentally friendly alternatives such as H_2O, ethanol or novel modern solvents that are being studied, such as ionic liquids (ILs) or deep eutectic solvents (DES).

The increasing interest in the use of natural compounds in replacement of fossil fuel derivatives has led to a considerable increase in the interest of the application of DES for the extraction of bioactive compounds from lignocellulosic biomass (Cunha and Fernandes, 2018; Paiva et al., 2014). DES are compounds formed by combining a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) to yield a mixture with a melting point below those of either of the pure components. They are formed by mixing two or more non-toxic compounds that are also cheap, renewable and biodegradable, which form an eutectic mixture (Cunha and Fernandes, 2018; Zhang et al., 2012), which confers upon it unique properties. Some of their most relevant properties are good chemical stability, negligible vapour pressure, and tuneable solubility, among others (Cvjetko Bubalo et al., 2018). Due to their properties, DES are considered as green and designer solvents (Mbous et al., 2017), which has led to them becoming one of the most popular solvents, along with ILs. There are many different combinations for the synthesis of ILs and DES, which results in a very large family of compounds. This allows the adaptation of properties such as viscosity, polarity, melting point and solubility (Vekariya, 2017) in order to facilitate and optimise the extraction of the target compounds (Passos et al., 2014).

Among the different bioactive compounds belonging to the extractive fraction of biomass, flavonoids are gaining in importance. They are becoming popular as part of bark extractives because they are bioactive compounds, which means that they are capable of modulating different biological activities (Ortega and Campos, 2019). As a result of this property, flavonoids have a great number of benefits, such as anti-allergic, antioxidant, vasoprotective, and anti-inflammatory properties, among others (Kesarkar et al., 2009). Antioxidant capacity is one of the most important of these, as it prevents the oxidation process, allowing the protection of other compounds such as lipids, DNA and proteins in biological systems (Cvejić et al., 2017). Thanks to their properties, their application in different fields is increasing, from food to personal care industries, and even in the creation of new bio-based materials (Feng et al., 2013; Maimoona et al., 2011; Mármol et al., 2019).

Therefore, knowing the excellent potential of tree bark as a natural source of flavonoids, the combination of this and extraction with DES becomes attractive with the aim of more sustainable extraction processes. Hence, interest in flavonoids extraction from natural sources coupled with the enormous potential that DES have for the selective extraction of these compounds, has resulted in the recent growth of this area. It is important not to ignore the fact that the DES properties allow operating at lower temperatures with the consequent advantages that it can generate, from the protection of volatile compounds to the reduction of energy consumption.

The main objective of this work was to study the use of different DES as selective solvents for the extraction of flavonoid compounds from *Larix decidua* bark. For this purpose, different extractions were carried out, and the achieved results were compared in order to choose the best selective solvent from the point of view not only of the extraction yield, but also of the composition of the extracts. The extracts were characterised by measuring both, total flavonoid content and different antioxidant capacities (DPPH, ABTS and FRAP).

2. Material and methods

2.1. Raw material

Larix decidua (from now on 'pine') tree bark was supplied by the Errekondo Egur-Zerra company (Basque Country, Spain). After collection, the raw material was prepared to obtain a homogeneous batch as granules with a size of less than 0.5 mm. For this, the steps followed were drying at room temperature, cleaning and grinding using a Retsch Cutting Mill SM 100. The chemical composition of the used feedstock was reported in our previous work (Sillero et al., 2020). Briefly, pine bark was 2.0 wt% suberin, 20.0 wt% extractives, 25.7 wt% cellulose, 7.6 wt% hemicelluloses, 36.8 wt% total lignin, and 3.5 wt% ash.

2.2. Synthesis of deep eutectic solvents

For the selective extraction of flavonoids, two DES were selected, choline chloride:urea (1:2) (DES 1) and choline chloride:1,4-butanediol (1:2) (DES 2). In both cases, the same synthesis method was used. Briefly, the reagents were dried under vacuum overnight, at room temperature. Then choline chloride (ChCl) was mixed with the chosen HBD (urea or 1,4-butanediol) in a ratio of 1:2 (ChCl:HBD). Then, the mixture was heated at 80 °C under constant stirring for 2 h, whereupon a clear and homogeneous liquid was obtained.

2.3. Deep eutectic solvents characterisation

The successful synthesis of both DES was confirmed by studying their structural characteristics using Nuclear Magnetic Resonance (NMR), ^{1}H NMR and ^{13}C NMR, and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR).

ATR-FTIR was collected using a Perking Elmer Spectrum Two spectrometer with a Universal Attenuated Total Reflectance accessory. The defined resolution was 4 cm^{-1} with 12 scans working in the range 700-4000 cm^{-1}.

The NMR spectra were recorded at 30 °C on a Bruker Ultrashield 400 MHz equipped with a z gradient BBI probe. Typically, 40 mg of sample were dissolved in DMSO-d_6. 2D-NMR (HSQC) spectra were recorded with a relaxation delay of 1.43 over 32 scans. The spectral widths were 5000 and 25,000 Hz for the ^{1}H and ^{13}C dimensions, respectively. For NMR acquisition, Top Spin software was

employed, and for further data processing, Mestre Nova (v.9.1) was used.

2.4. Extraction method

The conventional extractions were carried out in an orbital shaker with temperature control (Heidolph Unimax 1010 + Heidolph Incubator 1000) using a different mixture of DES/H_2O (0, 25, 50 and 75 wt%) as solvent. The operation conditions were 58 °C for 94 min, which were based on our previous work about extractions from pine bark (Sillero et al., 2018). These conditions were selected to avoid the degradation/evaporation of the DES. For this purpose, the work conducted by Delgado-Mellado et al. (2018) on the thermal stability of different DES was considered. They observed that the weight loss of the studied DES, in the worst case, was lower than 3% after 2 h at 70 °C. Thus, it confirms that the chosen parameters are suitable. Furthermore, it was also noted that the selected working conditions are within the range of those that have been used by other authors for the extraction of flavonoids with DES (Dai et al., 2013; Škulcová et al., 2018; Wan et al., 2019).

After the extractions, the solid and liquid phases were separated by vacuum filtration. Afterwards, the solids were washed with abundant distilled water, and air-dried. Finally, the extraction yields were calculated gravimetrically with Equation (1).

$$Extraction\ yield\ (\%) = 100 - \left[\left(\frac{W_{dried\ solid\ without\ extracts}\ (g)}{w_{dry\ bark}\ (g)}\right) x\ 100\right] \quad (1)$$

The results were expressed as the mean ± standard deviation of the three performed measurements. Analysis of variance (ANOVA) in IBM SPSS Statistic 24 software was used to conduct the statistical analysis of the extraction yield. For the significance study, Tukey's range test was used, considering statistically significant values those with p-values < 0.05.

The pH of all the used DES/H_2O mixtures were measured with a pH-2005 SELECTA. In addition, the extractions were compared to the polarities of the DES using the solvatochromic polarity scale described by Kamlet and Taft. This method is built on three solute-solvent interactions: hydrogen bond donating ability, α (Taft and Kamlet, 1976); polarisability, π* (Kamlet et al., 1977); and hydrogen bond accepting ability, β (Kamlet and Taft, 1976).

2.5. Chemical characterisation of the solid phase after the extraction

The remaining clean and dried solid after the extractions were submitted to a quantitative acid hydrolysis (QAH) (NREL/TP-510-42618) in order to quantify their glucan, lignin, and hemicelluloses content according to the methodology reported by Sillero et al. (2019).

2.6. Bark extracts characterisation

The characterisation of the extracts was carried out directly on the liquid phase obtained after separation by filtration from the solid phase. This means that the characterised extracts were a mixture of water, DES and extracted compounds. The total flavonoids content (TFC) was determined by the spectrophotometry technique reported by Blasa et al. (2006), following the procedure described by Sillero et al. (2019). Catechin was used as a standard, and the results were expressed as catechin equivalents (CE)/g of dried bark extract.

The potential of the bark extracts was studied by measuring their antioxidant capacity using DPPH, ABTS and FRAP assays. DPPH is a radical scavenging assay (Brand-Williams et al., 1995), FRAP measured the ferric reducing power (Benzie and Strain, 1996), and ABTS determines the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) equivalent antioxidant capacity (Re et al., 1999). All of these spectrophotometry measurements were performed with a Jasco V-630 UV-VIS spectrophotometer according to the procedure explained by Sillero et al. (2018). Trolox was used as a standard in the three methods, and the results were reported as mg of Trolox equivalent (TE)/g of dried bark extract.

Finally, in order to have a better comprehension of the structure of the extracts, the dried extracts were characterised by ATR-FTIR following the methodology described in section 2.3.

3. Results and discussion

3.1. DES characterisation

The DES were characterised by ^1H and ^{13}C NMR spectroscopy. The images of the spectra are provided in the supplementary materials, while the assignment is summarised below.

Peak assignment of DES 1was performed according to the structural data of other authors (D'Agostino et al., 2011; Delso et al., 2019). The peaks of the ^1H NMR spectrum of DES 1 are assigned as follow; δ_H (270 MHz, DMSO-d^6)/ppm: 3.25 (s, 9H, NCH3), 3.39 (s, 2H, NCH2CH2) 3.42 (t, 2H, CH2 CH2O), 3.83 (m, 2H, CH2O), 5.51 (s, 8H, CNH2). The peaks of the ^{13}C NMR spectrum of DES 1are assigned as follows; δ_C (68 MHz, DMSO-d^6)/ppm: 54,0 (t, 3C, NCH3), 55,3 (s, CH2CH2O), 67,7 (s, N CH2CH2), 160.1 (s, 2C, NH2CONH2).

Peak assignment of DES 2 was according to the structural data of another author (Delso et al., 2019). The peaks of the ^1H NMR spectrum of DES 1 are assigned as follows; δ_H (270 MHz, DMSO-d^6)/ppm: 1.42 (t, 4H, $CH_2CH_2CH_2$) 3.14 (s, 9H, NCH3), 3.35 (t, 8H, CH2CH2O) 3.42 (t, 2H, NCH2 CH2), 3.83 (m, 2H, CH2CH2O), 4.47 (s, 4H, CH2OH), 5.59 (t, 1H, CH2OH). The peaks of the ^{13}C NMR spectrum of DES 2 are assigned as follow; δ_C (68 MHz, DMSO-d^6)/ppm: 29.5 (t, $CH_2CH_2CH_2$) 53,5 (t, 3C, NCH3), 55,3 (s, CH2CH2O), 61.2 (s, 2C, OCH2CH2) 67,7 (s, NCH2CH2).

ATR-FTIR analysis of the DESs was also performed. Fig. 1 shows the spectra of the two DES, where structural differences can be

seen. The band assignment is based on the assignments given by other authors (Ma and Row, 2017; Meng et al., 2018; Piasek and Urbanski, 1962), and a summary of this assignment is given in Table 1.

As shown in Fig. 1 and Table 1, there are many similarities between the structures of the two synthesised DES. In fact, many of the bands identified correspond to those of ChCl, which, although when the DES is synthesised, loses some of its characteristic bands, it has been demonstrated that there are others that remain regardless of the HBD (Delgado-Mellado et al., 2018). The bands typically preserved are those at 2900-2850 and 1484-1420 cm^{-1} which correspond to vibrations of the alkyl group, with the most pronounced band at 1485 cm^{-1} corresponding to the bending of the CH_2 group; 1200-800 cm^{-1} attributed to N–H stretching and C–N symmetric stretching; and the band detected at 3200 cm^{-1} assigned to the hydroxyl groups. When the DES is formed, these bands continue, but other bands appear or the existing ones are affected in intensity when the DES is generated, which means that the spectra of the different DES are not equal.

The main differences between the spectra of the two DES are in the ranges 3500-2500 cm^{-1} and 1750-1250 cm^{-1}. The DES 1 has, apart from the peak at 3300 cm^{-1} associated with –OH vibration of the pure choline chloride, another at 3189 cm^{-1} which is identified as –NH stretching, coming from urea. Furthermore, it also has two high-intensity peaks in the range of 1600–1700 cm^{-1} that correspond to the stretching of the –CN of urea. DES 2, however, has the bands associated with –CH stretching with higher intensity (2900-2700 cm^{-1}), due to the presence of 1,4-butanediol. The rest of the bands are characteristic of choline chloride, so they are similar, although they vary in intensity.

These spectra together confirm that the syntheses were completed successfully, and that the obtained compounds were the desired ones.

3.2. Flavonoid extraction with DES

In this work, the extractions were carried out using the different DES as additives in combination with water. In general, water is considered the most sustainable option for the extraction of different compounds from lignocellulosic biomass, although it is not always suitable when use alone. This is the case for flavonoids, whose properties lead them to exhibit limited solubility (Ali et al., 2019). Therefore, in recent years many studies have been carried out to improve the extraction of flavonoid compounds by using water/organic solvent mixtures (Li et al., 2018; Sillero et al., 2018; Yu et al., 2017; Zhao et al., 2017). In this work, however, these organic solvents have been replaced by greener solvents such as DES. We selected three compositions, 25 wt%, 50 wt% and 75 wt% DES in water in order to be able to compare to pine bark extractions with ionic liquid/water mixtures that had previously used these compositions (Sillero et al., 2020). We did not conduct the extractions with dry DES, since these are solid at room-temperature.

Even though water has limited chemical affinity for flavonoid compounds, its use with DES decreases the viscosity of the solvent, facilitating the extraction (Dai et al., 2013). However, viscosity is not the only parameter that varies, since polarity is also affected, which is directly related to the extraction capacity of these compounds. This parameter is described as the sum of the total possible interactions that occur between the solute and the solvent. Thus, the polarities of both DES, and of their mixtures, would not be equal, as can be seen in Table 2 where the solvatochromic parameters calculated for both DES are summarised.

The parameter α is principally influenced by the HBD, urea and 1,4-butanediol, with some additional contribution from the –OH functionality of the choline cation. The values of π* are affected by both HBD and HBA, and DES 2 had the highest value. β parameter is more dependent on the HBA, which in both of these cases is the chloride ion. However, it is known that although the hydrogen bond acceptor property of the DES arises principally from the anion, it is moderated by the HBD. DES 1 has the lowest value, because urea is

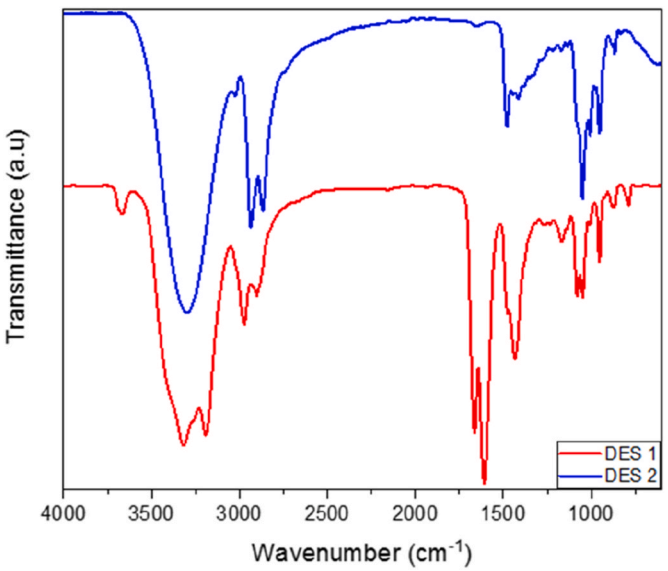

Fig. 1. ATR-FTIR spectra of the two DES, choline chloride:urea (DES 1) and choline chloride:1,4-butanediol (DES 2).

Table 1
FTIR spectra band general assignment.

Wavenumber (cm^{-1})	DES 1	DES 2	Band assignment	Reference
3317	X	x	symmetric NH_2 stretching	Ma and Row (2017)
3200	X		-OH stretching	Delgado-Mellado et al. (2018)
2970–2850	X	x	-CH stretching from an alkyl group	Delgado-Mellado et al. (2018)
1662–1606	X		-NH deformation vibration	Du et al. (2016)
1485–1420	X	x	-CH stretching from an alkyl group	Delgado-Mellado et al. (2018)
1200–800	X	x	N–H stretching and C–N symmetric stretching	Delgado-Mellado et al. (2018)

Table 2
Kamlet-Taft coeficients of the pure solvents used in this work, measured using the dyes N,N-diethyl-4-nitroaniline, 4-nitroaniline,and Reichardt.

Abbreviation	Solvent	α	β	π*	Reference
H_2O	H_2O	1.23	0.47	1.14	Jessop et al. (2012)
DES 1	ChCl:Urea	1.42	0.50	1.14	Florindo et al. (2018)
DES 2	ChCl:1,4-butadienol	0.65	0.79	1.74	Harris (2008)

the more basic compound of the two HBD. In order to determine the polarity of the mixtures used in this work, an estimation was made, taking the water values (Table 2) as a reference, which will be affected to a greater or lesser degree depending on the DES percentage used in each mixture. This discussion will be held in combination with the discussion of the extraction yields.

In this work, seven different solutions, were prepared and tested for the extraction of pine bark, and Table 3 shows the determined extraction yields. These results range from 9% to almost 22% of the dry weight of the bark. The lowest value measured was recorded for water as solvent, while the highest yield was obtained for DES 1/H_2O (75 wt%). All the extractions carried out are significantly different from the one with water as shown in Table 3.

The data clearly show that the concentration of DES in the DES/H_2O mixture has a significant effect, with lower extraction yields measured for the DES/H_2O mixtures with the lowest DES concentration. According to the data, the pH of the mixtures had no direct influence on the extraction yield, while the percentage of water has a direct influence. However, it can also be seen that the difference in the extraction yields for the 50 wt% and 75 wt% DES/H_2O mixtures are much less than the difference between these and the 25 wt% DES/H_2O mixtures. Wan et al. (2019) have suggested that a proportion of water higher than 70% in the mixture decreases the extraction yield due to the destruction of the DES structure, which may be responsible for the results described here.

Looking at the measured extraction yield for the different DES mixtures, it can be seen that there are only small differences between the DES 1/H_2O and DES 2/H_2O mixtures at the same concentration. Hence, no clear trend can be observed in these small differences. DES 1 has values for both β and π* that are very close to those of water, but has a higher value for α (1.42) than does water (1.23). In this case, the higher yield can be tentatively attributed to the higher α value. However, in the case of DES 2 all parameters are different to those for water, β and π* increased while, contrary to DES 1, α decreases. Thus, there is no clear trend on how the different polarity parameters can affect the extraction yield of the pine bark in the studied conditions. The increased extraction of flavonoid compounds is likely the result of many interactions between these and the DES.

A significant increase in the extraction yield is observed when comparing the yields obtained in this work with those reported with conventional solvents (EtOH/H_2O) for the same raw material. Sillero et al. (2018) carried out the optimisation of three different extraction methods, conventional, assisted by ultrasonic and assisted by microwave (8.2%, 6.1% and 8.3% extraction yield, respectively). These results are greatly improved in this work, as well as those obtained for the same feedstock using simultaneous microwave-ultrasound assisted extraction (15.7% extraction yield) (Sillero et al., 2020).

Looking at other works that investigated the use of DES to obtain flavonoids, the first to be mentioned is by Škulcová et al. (2018), who carried out the extraction of Spruce bark using nine different DES. The extraction yields reported in this study ranged from 11.4% (ChCl:glycerol, 1:1) to 27.7% (ChCl:tartaric acid (1:1), so it can be said that all our results are within similar range. In the case of the results reported by Haz et al. (2018) for the same raw material using three different DES, the reported extraction yield was lower. The results varied between 11.40% (ChCl:glycerol, 1:1) and 14.68% (ChCl:malic acid, 1:1).

Table 3
Extraction yield determined for the different DES/H_2O mixtures, and results of the characterisation of the extract.

Solvent	[DES] (wt.%)	pH	Yield* (wt.%)	TFC (mg CE/g DBE)	DPPH (mg TE/g DBE)	ABTS (mg TE/g DBE)	FRAP (mg TE/g DBE)
H_2O	0	5.80	9.3 ± 0.2^{a}	34 ± 2	22.3 ± 0.3	106 ± 2	31 ± 4
DES 1/H_2O	25	9.49	$16.2 \pm 0.7^{b,c}$	159 ± 7	215 ± 20	391 ± 17	98 ± 2
	50	9.77	19.4 ± 0.1^{d}	305 ± 9	297 ± 17	593 ± 16	155 ± 8
	75	10.16	21.9 ± 0.8^{e}	275 ± 1	215 ± 4	637 ± 45	120 ± 2
DES 2/H_2O	25	4.63	16.5 ± 0.7^{c}	376 ± 3	460 ± 17	850 ± 54	309 ± 7
	50	5.70	$20.0 \pm 0.5^{d,e}$	376 ± 12	453 ± 8	736 ± 22	314 ± 6
	75	5.29	$20.4 \pm 0.1^{d,e}$	383 ± 7	452 ± 19	799 ± 19	289 ± 11

DBE: dried bark extract.
*Superscript letters depict significant differences (Tukery test, $p < 0.05$)).

Once the overall extraction yield had been studied, the next step was to confirm that the extracted compounds belonged only to the extractive fraction of the lignocellulosic biomass. For this purpose, chemical characterisation of the solids remaining after extraction was performed by QAH. In this way, the variation of the hemicelluloses, cellulose (expressed as glucan) and acid-insoluble lignin content of the solids was studied (Table 4).

The first thing to point out is that no glucan solubilisation was observed in any of the performed extractions. The evolution of the solubility in each DES/H_2O mixture revealed that there was practically no variation between them (variation of ± 0.8 wt%), nor in comparison with the extraction carried out only with water. This confirms that in the studied conditions there is no glucan solubilisation. Similarly, there is also no solubilisation of hemicelluloses, with a maximum variation of 0.8 wt%.

Acid insoluble lignin (AIL) appears to be the main component of the solid fraction, and this is the fraction in which most variation has been measured. It is observed that the highest value was determined for the solid treated with water, 50.2 wt%, while the values for the rest of the solids were in the range of 40–45 wt%. In the comparison, it is observed that for the extractions carried out with the DES/H_2O mixtures, the solubilisation of the AIL increases with the increase of the DES concentration. However, these differences in the results may be due to the solubilisation of suberin or extracts since the measurement of AIL can be affected by these compounds (Sluiter et al., 2012). Therefore, it cannot be stated with certainty that solubilised lignin is present in the extractions performed under these conditions.

3.3. Flavonoid quantification

It has been confirmed that the extraction yield observed for the DES/H_2O mixtures is considerably higher than that reported for water/organic solvent mixtures for pine bark, so the next step was to verify the selectivity of the extraction. For this purpose, the total flavonoid content (TFC) of the liquid phase was measured. Table 3 illustrates that water did not extract flavonoids at all, which confirms the poor affinity of these compounds for water (Meng et al., 2018).

The TFC varies from 159 to 383 mg CE/g dried bark extract. In general, the amount of flavonoids obtained with the DES 2 mixtures are more than 25% higher than those obtained with DES 1. The highest TFC was determined for DES 2 (75%), although the difference with the other two DES 2 mixtures was slight. On the other hand, there were large differences in the TFC values for the DES 1/H_2O mixtures at different concentrations. It is observed that the worst values were obtained with the lowest DES concentrations. This could indicate that higher DES concentration favours flavonoid extraction up to a point at which no further improvement is achieved.

Comparing the TFC values obtained in this work with the values reported by other authors, in general it can be said that good results have been achieved. The values determined with DES 2/H_2O are in the range of the ones reported for acetone/H_2O extracts from *Pinus durangensis* (379 mg CE/g extract) (Rosales-Castro et al., 2017), and the ones reported for hydroalcoholic extracts from *Qercus sideroxyla* (386 mg CE/g extract) (Soto-García and Rosales-Castro, 2016). However, the values measured with DES 1/H_2O are notably lower. Comparing the results obtained here with those obtained for the same raw material with EtOH/H_2O, by both conventional and different intensification methods (ultrasound, microwave and simultaneous microwave-ultrasound assisted extraction), even the values determined for DES 2 are lower, although not greatly so. Sillero et al. (2018) reported values between 412 and 430 mg CE/g dried bark extract for conventional, ultrasound assisted and microwave assisted extractions. While, Sillero et al. (2020) reported values of 433 mg CE/g dried bark extract for the EtOH/H_2O extracts obtained by simultaneous microwave-ultrasound assisted extraction. The difference of the TFC may be due, on the one hand, to the different viscosities of the applied solvents, and on the other hand, to the solvent's polarity. It is also important to consider the possible steric hindrance of DES, especially of DES 2.

Studying different works performed for the extraction of flavonoid compounds from different lignocellulosic materials with DES, both Wang et al. (2019) and Cui et al. (2018) conclude that ChCl:1,4-butanediol was the best. Although in the case of Ciu et al. (2018) the best ratio was 1:3, while in the case of Wang et al. (2019) the best ratio was 1:2. This is in accordance with the results obtained here, where among all the extractions done with DES, the best TFC was obtained for DES 2 (75%) extract. The use of 25% water to facilitate the extraction of flavonoid compounds is in accordance with that reported by Wang et al. (2019). Ma and Row (2017) studied the extraction of three different flavonoids from *Herba Artemisiae Scopariae* using different ILs and DES as solvents, including the two DES studied here. The best measured values for DES were obtained with ChCl:formic acid (10234.43 μg/g rutin, 1032.23 μg/g quercetin, and 211.87 μg/g scoparon), although both DES 1 and DES 2 extracted high contents of these flavonoids, with DES 2 showing slightly better results.

Considering all the above, it can be concluded that the extraction yield is not associated with the flavonoid extraction. According to Table 3, the best extraction yield was obtained with DES 1 (75%), however, this is not consistent with the highest TFC. This may be a consequence of a lower selectivity of these solvents for two reasons. On the one hand, the use of water in the mixtures may have resulted in the extraction of other non-flavonoid compounds, since it alone extracts substances from pine bark as it is shown in Table 3.

Table 4
Chemical composition of the pine bark after the extractions (all results are expressed as wt.%).

Solvent	[DES]	Acid-insoluble lignin	Glucan	Hemiceluloses
H_2O	0	50.2 ± 3.2	27.7 ± 0.6	11.1 ± 0.1
DES 1/H_2O	25	45.4 ± 1.4	27.6 ± 0.3	11.7 ± 0.1
	50	41.3 ± 1.3	27.7 ± 0.1	11.2 ± 0.5
	75	40.38 ± 4.7	28.0 ± 0.2	11.0 ± 0.7
DES 2/H_2O	25	45.1 ± 1.9	27.8 ± 0.4	11.1 ± 0.3
	50	43.5 ± 0.3	27.7 ± 0.3	11.0 ± 0.9
	75	41.8 ± 0.6	27.2 ± 0.1	10.9 ± 0.1

On the other hand, it is important to mention that DES 1 has also been studied for the delignification of different lignocellulosic materials (Espinoza-Acosta et al., 2014; Kalhor and Ghandi, 2019; Prado et al., 2018). This indicates that the use of DES 1 mixture for flavonoid extraction may also solubilise part of the lignin. Therefore, reporting a high extraction yield whereas TFC would not be consistent. This is confirmed by the decrease of the AIL content measured in the solids after extraction (Table 4).

3.4. Characterisation of bark extracts: antioxidant capacity

A further point to consider for the possible application of the extracts is their antioxidant capacity. Three antioxidant capacity measurements have been performed in this work, providing a more accurate idea of the capacity of these extracts. The values measured for the studied antioxidant capacities are different for the same extract, which is normal because they measure different aspects.

Table 3 shows the values determined for the 3 measured parameters, and the first thing to point out is the low antioxidant capacity of the aqueous pine bark extracts. This is in line with the low TFC, which confirms the benefit of the use of DES for the extraction of flavonoid compounds. The range of values measured for the DPPH of the different tested extracts were between 215 and 460 mg TE/g dried bark extract. The highest determined value was for DES 2 (25%) extract, but there was no significant difference with the other two DES 2 extracts. The extracts obtained with DES 1 all exhibited lower DPPH values, in accordance with their lower TFC. In the case of ABTS, as with DPPH, the extracts obtained with DES 2 had the highest results, although in general all ABTS values were higher than DPPH values. The best value determined was the one measured for DES 2 (25%) extracts, 850 mg TE/g dried bark extract. Finally, regarding the FRAP assay, DES 1 extract give the lowest antioxidant capacities, with the worst, as for the other antioxidant capacities, were obtained with 25 wt% DES 1/H_2O. The values in this case are especially low, since they did not even reach 100 mg TE/g dried bark extract. This is consistent with the fact that DES 1/H_2O mixtures are the ones with the lowest flavonoid extraction, which suggests that this solvent is not very selective for the extraction of flavonoids.

Comparing the values presented in this study with those previously obtained for pine bark using EtOH/H_2O as solvent (Sillero et al., 2018), it can be concluded that all the measured values here are in the range (677–906 mg TE/g dried bark extract), except for the 25 wt% DES 1/H_2O extract. Regarding the FRAP assay, however, only the extracts obtained with DES 2 approaches the results obtained with EtOH/H_2O (330–390 mg TE/g dried bark extract).

Additionally, a cautious comparison has also been conducted with the results recorded for other raw materials. Bibi Sadeer et al. (2019) obtained methanolic extracts from 3 different tree barks, where the lowest ABTS and DPPH values were measured for *Zanthoxylum gilletii* (178 and 82 mg TE/g extract, respectively). These values are far exceeded in this work. In the case of DPPH, values have been reported for *Macaranga hurifolia* and *Sterculia tragacantha* close to 495 mg TE/g dried bark extract, which is in the range of those calculated for the extracts obtained with DES 2/H_2O mixtures (452–460 mg TE/g dried bark extract). Analysing the values measured by Bibi Sadeer et al. for FRAP, the value reported for the methanolic extract of *Zanthoxylum gilletii* (163 mg TE/g extract) was lower than the values reported for DES 2/H_2O extracts.

In the work conducted by Tanase et al. (2019), *Fagus sylvatica* bark extracts obtained with different solvents by microwave-assisted extraction were characterised. The ABTS antioxidant capacity reported for *Fagus sylvatica* bark extracts obtained with 80% ethanol in water was 472 mg TE/g dried extract. These results were exceeded in all experiments in this work except for the DES 1 extract (25%). All the results reported for DPPH and ABTS were better than those determined in this work.

A comparison of the antioxidant capacities of *Chrysophyllum perpulchrum* extracts obtained using different solvents by Baloglu et al. (2019) shows that the highest DPPH value obtained was for MeOH extracts (73.23 mg TE/g extract). This value is significantly lower than those obtained here for pine bark extracts (Table 3). Something similar occurs with the aqueous extracts for ABTS antioxidant capacity (491 mg TE/g extract), where only the DES 1 (25%) extracts presented a lower value.

Considering the aforementioned, it can be concluded that the use of DES promotes the extraction of antioxidant compounds, especially the use of DES 2.

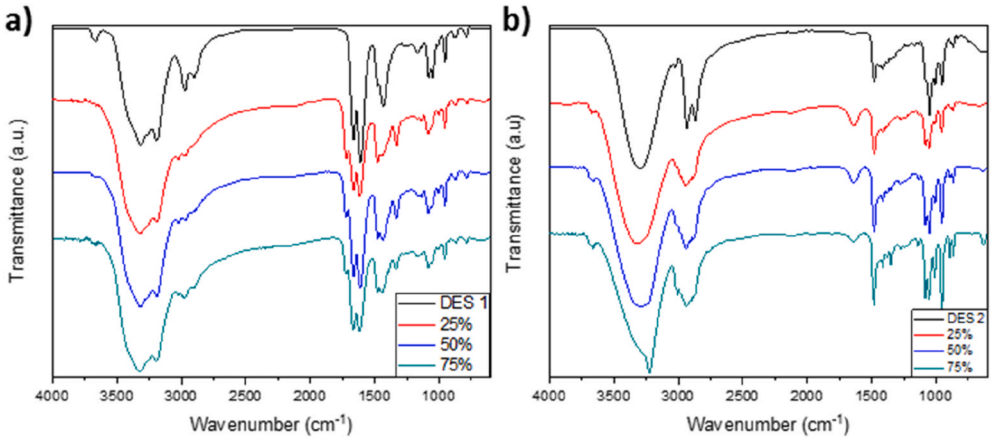

Fig. 2. ATR-FTIR spectra of different bark extracts. a) Extracts obtained with different DES 1 concentrations. b) Extracts obtained with different DES 2 concentrations.

3.5. Structural characterisation of extracts

The presence of flavonoid compounds in the different extracts was confirmed by ATR-FTIR analysis. The following figures show the extracts with different concentrations of solvents compared to the spectrum of the pure DES used as a solvent. In the Fig. 2 it can be seen how the intensities of the different bands change depending on the concentration of solvent used, as well as the appearance of new bands.

In the two studied cases, a significant increase in intensity is observed in the band attributed to –OH stretch vibration in phenolic and aliphatic structures (between 3400 and 3300 cm^{-1}). Fig. 2b of DES 2 extracts shows that the band at wavenumber 1630 cm^{-1} undergoes a considerable increase in intensity, which is assigned to the valence vibrations C═O, typical of flavonoid compounds (Trifunschi et al., 2015). Thus, the extraction of these compounds is confirmed. In the case of DES 1, as shown in Fig. 2a, instead of the typical C═O band of the flavonoids, another band appears at a wavenumber of 1705 cm^{-1}. This band is related to the presence of lignin, since it corresponds to the stretching vibration of non-conjugated carbonyl groups from the aromatic lignin skeleton (Boeriu et al., 2004). This confirms the capacity of this solvent to solubilise lignin, as it was mentioned in above.

4. Conclusions

Two DES have been successfully synthesised in this work to be used as green solvents for the extraction of bioactive compounds. They have been used as additives in aqueous mixtures to enhance the selective extraction of flavonoid compounds from pine bark.

In this work, it is confirmed that the use of aqueous mixtures of DES can be an alternative solvent for flavonoids extraction from pine bark, since all the cases studied showed an improvement in extraction yield in comparison with aqueous extraction. Moreover, the extraction yields have been improved compared to the results obtained with conventional solvent ($EtOH/H_2O$). This further demonstrates the potential of DES for this use. However, for TFC, only DES 2 mixtures achieved results similar to those reported with conventional solvents. Furthermore, this work verifies that DES 1 cannot be considered a selective solvent for flavonoids, since although the highest extraction yield is obtained with DES 1 (75%), the TFC content is low, as well as the antioxidant capacity of the extracts. This can be explained by the solubilisation of lignin when DES 1 is used, which is verified in the ATR-FTIR results.

DES $2/H_2O$ (75% wt%) was selected as the best solvent due not only to its good ability to extract flavonoids, but also for the high antioxidant properties of the extract. The good flavonoid extraction capacity was confirmed by the ATR-FTIR analysis of the extracts, which showed a large increase in the band typically assigned to the flavonoids at 1630 cm^{-1}. Consequently, it can be confirmed that biologically active extracts have been obtained. This is very suitable for their use in different applications, from food industry to cosmetics, or biologically active bio-based materials. Nevertheless, further characterisation of the obtained compounds and their purification prior to their application should be carried out before their use.

CRediT authorship contribution statement

Leyre Sillero: Methodology, Investigation, Visualization, Writing – original draft, Preparation. **Raquel Prado:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Tom Welton:** Resources, Validation, Writing – review & editing. **Jalel Labidi:** Resources, Supervision, Writing – review & editing, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2021.100544.

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