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Investigation of Total Phenolic Content and Antioxidant Activities of Spruce Bark Extracts Isolated by Deep Eutectic Solvents

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Abstract: Extracts from spruce bark obtained using different deep eutectic solvents were screened for their total phenolic content (TPC) and antioxidant activities. Water containing choline chloride-based deep eutectic solvents (DESs) with lactic acid and 1,3-propanediol, 1,3-butanediol, 1,4-butanediol, and 1,5-pentanediol, with different molar ratios, were used as extractants. Basic characteristics of the DESs (density, viscosity, conductivity, and refractive index) were determined. All the DESs used behave as Newtonian liquids. The extractions were performed for 2 h at 60 °C under continuous stirring. TPC was determined spectrophotometrically, using the Folin-Ciocalteu reagent, and expressed as gallic acid equivalent (GAE). The antioxidant activity was determined spectrophotometrically by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The TPC varied from 233.6 to 596.2 mg GAE/100 g dry bark; radical scavenging activity (RSA) ranged between 81.4% and 95%. This study demonstrated that deep eutectic solvents are suitable solvents for extracting phenolic compounds from spruce bark.

Keywords: deep eutectic solvents; phenolic compounds; antioxidant activity; spruce bark; extraction

1. Introduction

Valorization of biomass, bio-waste, and food-related wastes (hereinafter biomass), including extraction of value-added compounds from these sources, represents a dynamically developing area of research and technology [1–34]. In the field of biomass valorization, a number of green extraction methods have been applied, and their results and usability have been reviewed (for the latest reviews see, [1,2,13,16–20]).

A substantial body of research has focused on the new modes of extraction and refining processes during the last decades. Biomass contains many exceptional and especially health-promoting substances. The most important potential uses of compounds extracted from biomass includes pharmaceutical and biomedical applications, and applications in the food industry as additives and functional substances, as well as nutraceuticals used to enhance food quality or in gastronomy [1–3].

In the last decade, several research teams have published data focused on the purposeful processing of many kinds of biomass. Examples include *Pseudowintera colorata (horopito)*, a plant native to New Zealand, herbal tea [21], olive, soy, peanuts, corn, and sunflower oil [22], olive cake, onion seed, and by-products from the tomato and pear canning industries [23], pomelo [24], rice straw [25], wood,

straw, pulp [26–28], bark of spruce and other tree species [29–31], corn stover, switchgrass, saffron wastes [32], *Miscanthus* [33], and coffee and cocoa by-products [34]. In the field of biomass pretreatment and delignification with DESs, several DESs have been investigated, and the results of their usability have been examined [13]. One of the main tasks of this industry is the separation of the lignin and the cellulosic fractions of the biomass. There are several methods and procedures to change the composition of the original lignocellulosic matrix aimed at eliminating one of its components (lignin or polysaccharides), thus obtaining new products (pulp, microcrystalline cellulose, nanocellulose etc.), and valorizing the biomass. These include processes such as solubilisation, extraction, fractionation, deconstruction, delignification, and post-delignification [1,13,28,33–41]. Value-added substances and compounds are isolated from biomass by extraction techniques using predominantly water and common organic solvents, and, to a minor extent, eco-friendly green solvents represented by deep eutectic solvents [13]. To reach high extraction yield of target compounds, various extraction techniques have been developed, the most frequently used being supercritical fluid extraction, pressurized liquid extraction, and ultrasound-assisted extraction [7].

One of the most important classes of extractable target compounds is represented by polyphenols, which exhibit, *inter alia*, antioxidant properties, mainly due to their radical scavenging activity [11,12,14,15,42–49]. These substances present exceptional properties, exerting antagonist, antiallergic, antiangiogenetic, antiatherosclerotic, anticancer, antidiarrheal, antihypertensive, anti-inflammatory, antimicrobial, antimutagenic, antimycotical, antineoplastic, antioxidant, antiproliferative, antiseptic, antitumor, antiviral, cytotoxic, estrogenic, fungicidal, hepatoprotective, insecticidal, neuroprotective, and pharmacokinetic activities [3]. In vitro antioxidant activity of bark extract is described by Selvasundhari et al. (2014) [50] and Patrick et al. (2016) [51]. The effectiveness of the extraction is quantified through total extract yield, total phenolic content (TPC), total flavonoid content, and total antioxidant capacity [52]. Particular attention is devoted to *trans*-resveratrol (*trans*-RSV), which is considered a powerful compound capable to improve health and prevent chronic diseases in humans, protecting against some neurodegenerative diseases, obesity and diabetes, high blood pressure, as well as cancer and osteoporosis [7]. In Table 1 the total phenolic content and the antioxidant activity for spruce and pine bark extracts obtained by supercritical fluid extraction, pressurized liquid extraction, ultrasound-assisted extraction, soxhlet extraction, and ohmic heating extraction techniques are reported.

Table 1. Total phenolic content (TPC) and antioxidant activity of spruce and pine extracts.

Extraction	TPC (mg GAE/g Dry Extract)	ABTS (mg TEs/g Dry Extract)	FRAP (μmol $FeSO_4 \cdot 7H_2O$/g Dry Extract)	Ref.
SFE_10 % conc. of ethanol, *v/v*	0.77	2.48	8.31	[7]
SFE_20 % conc. of ethanol, *v/v*	1.24	3.08	10.01	[7]
SFE_40 % conc. of ethanol, *v/v*	2.50	5.29	25.49	[7]
PLE_ethanol	33.45	69.87	389.10	[7]
PLE_ethanol	46.32	257.11	506.10	[7]
UAE_ethanol	54.97	128.47	580.25	[7]
SFE_ ethanol	6.11–11.30	0.68–0.79 *		[53]
Soxhlet extraction_n-hexane		8.3 *		[54]
Soxhlet extraction_n-hexane		4.5 *		[54]
ASE_n-hexane		15 *		[55]
Ohmic heating extraction_water ***		136–156 **		[56]

Table 1. *Cont.*

Extraction	TPC (mg GAE/g Dry Extract)	ABTS (mg TEs/g Dry Extract)	FRAP (µmol $FeSO_4 \cdot 7H_2O$/g Dry Extract)	Ref.
Ohmic heating extraction_50 % conc. of ethanol, *v/v*, ***		807–990 **		[56]
Extraction_50 % conc. of ethanol, *v/v*, ***		394–444 **		[56]
Extraction_water, ***		111–120 **		[56]

ASE: accelerated solvent extraction; PLE: pressurized liquid extraction; SFE: supercritical fluid extraction; UAE: ultrasound-assisted extraction; TEs: Trolox equivalent; $FeSO_4 \cdot 7H_2O$: ferrous sulfate heptahydrate; ABTS: 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: ferric reducing antioxidant power; TPC: total phenolic content, * - mmol TEs/g dry extract, ** - µmol TE/ g dry bark; *** - pine bark.

This paper is devoted to three mutually overlapping research topics: softwood bark as the object of processing; polyphenols as the extracted antioxidants; and deep eutectic solvents as extractants.

The focus on softwood bark is explained by the fact that the annual volume of harvested soft woods in Central and Northern Europe is about 25×10^7 m^3, of which ca. 10% is bark, which is currently disposed of or burned for energy recovery. Thus, Norway spruce (*Picea abies* [*Karst*.]) bark can be regarded as a largely available source of condensed polyphenols in Europe [5–10]. Meanwhile, the rationale behind the choice of polyphenols lies in their mentioned properties and usability.

Deep eutectic solvents (DESs) are mixtures of two or more components—a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA)—which can bond with each other to form a eutectic mixture having a lower final melting point relative to the melting points of the HBA and HBD [4]. From the practical point of view, it is an advantage if a formed DES is liquid at room temperature.

When the compounds that constitute the DES are exclusively primary metabolites, namely, amino acids, organic acids, sugars, or choline derivatives, the DESs are called natural deep eutectic solvents (NADESs). The term "low-transition temperature mixtures" is used for both types of eutectic mixtures (DESs and NADESs), as well as for liquids composed of natural high-melting-point starting materials, which are not eutectic. Common features of the mentioned solvents and slight differences between them are discussed elsewhere [13], in this work we will stick to the most frequently used term "deep eutectic solvents" (DESs).

Contrary to the majority of organic solvents, which are inflammable liquid substances with relatively high vapor pressure, low viscosity, frequently considerable toxicity for living organisms, and with a negative impact on the environment, DESs have attractive physicochemical properties, such as fire resistance, negligible vapor pressure, miscibility with water, and liquid state in a wide temperature range. Being multi-component systems, DESs offer significant advantages over conventional organic solvents; their structure may be modified by the selection of solvent-forming components, as well as by the molar ratio of the components participating in hydrogen bond formation. That is why their properties (e.g., freezing temperature, viscosity, conductivity, refractive index, density, and pH) are significantly influenced by the molar composition of the compounds in the mixture and can be purposefully modified or even optimized [13].

Various methods and conditions have been investigated to extract polyphenols from softwood bark, and, depending on the method applied, different TPC values have been reached for the same bark sample [43]. The effect of temperature was documented by Lazar et al., [57] who reached TPCs of 37.3 mg and 43.1 mg GAE/g spruce bark at 45 °C and 60 °C, respectively. Conde et al. [58] investigated the effect of temperature and pressure on the extractive yield and the total amount of phenolic compounds from maritime pine and beech wood under conditions of 10–25 MPa, 30–50 °C, supercritical CO_2 with 10% ethanol. The highest extraction yield (6.1 g extract/100 g wood) was reached at 30 °C and 15 MPa, and TPC 7.6 g GAE per 100 g extract at 50 °C and 25 MPa.

Jablonsky et al. [3] summarized the properties of 237 compounds from studies on coniferous bark extraction. The authors reviewed the activities of various substances isolated from the bark: cytotoxic (25 identified substances); antioxidant (26 substances); antibacterial (42 substances); anti-inflammatory (10 substances); antimutagenic (5 substances); pharmacokinetic (5 substances); pheromone (10 substances); and inhibitors (22 substances).

Škulcová et al. [29] applied different types of DESs to extract compounds from spruce bark. The extracts from spruce bark showed increased antioxidant activity compared with the corresponding pure DES. The polyphenols content in eutectic extracts ranged from 41 to 463 mg of gallic acid equivalent (GAE) per 100 g of extract. The highest levels of polyphenols were achieved using the following ChCl-based DESs: lactic acid (463 mg GAE/100 g extract); glycolic acid (398 mg GAE/100 g extract); malonic acid (209 mg GAE/100 g extract); tartaric acid (198 mg GAE/100 g extract); oxalic acid (191 mg GAE/100 g extract); citric acid (119 mg GAE/ 100 g extract); glycerol (82 mg GAE/100 g extract); maleic acid (52 mg GAE/100 g extract); and the lowest level of polyphenols using ChCl with malic acid extract (41 mg GAE/100 g extract). Chupin et al. [59] obtained 18.07 ± 3.82 mg GAE/g bark when extracting pine bark with 80% aqueous ethanol by MAE. The water/ethanol mixture extracted the highest content of phenolic substances (73.48 ± 1.84 mg GAE/g DM), followed by ethanol and then by water (63.38 ± 1.26 mg GAE/g DM and 50.09 ± 4.70 mg GAE/g DM, respectively) [60], where DM is dry matter. Jablonsky et al. [61] summarized the pharmacokinetic properties of biomass-extracted substances isolated by green solvents.

In this study, three-component systems with choline chloride, lactic acid, and water, as well as four-component systems comprising water, choline chloride, lactic acid in different combinations with 1,3-propanediol, 1,3-butanediol, 1,4-butanediol, or 1,5-pentanediol, with different molar ratios, were used to extract phenolic compounds from spruce (*Picea abies*) bark. The DESs used were first prepared and their physico-chemical properties described in Jablonsky et al. (2019) [28]. The novelty of this paper lies in two factors: a new source of antioxidant compounds was used (*Picea abies*); and DESs were used as green solvents to isolate antioxidant compounds. Moreover, antioxidant activity and total phenolic contents of different extracts from spruce bark were determined.

2. Materials and Methods

2.1. Chemicals

All reagents, standards, and solvents were of analytical grade. Choline chloride (ChCl) (≥ 98.0%), 1,3-propanediol (98%), 1,3-butanediol (≥ 99.5%), 1,4-butanediol (≥ 99.0%), 1,5-pentanediol (≥ 96.0%), Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical were purchased from Sigma-Aldrich (Germany). Lactic acid (LacA) 90.0% solution was obtained from VWR International (Bratislava, Slovakia). Choline chloride was dried under vacuum. The other chemicals were used as supplied without further purification.

2.2. Preparation of Deep Eutectic Solvents

The DESs were prepared by mixing and stirring the corresponding components in a water bath (60 °C; 30 min) to form a homogeneous liquid. Key information about the DESs used is summarized in Table 2. The main characteristics of the DESs are gathered in Table 3.

Table 2. Prepared deep eutectic solvents (DESs), molar ratios of their components, and viscosity.

Sample	Component A	Component B	Component C	Component D	Molar Ratio	Water Content (%)	Viscosity at 60 °C (mPa S)
DES1	ChCl	LacA	-	Water	1:2:0.96	5.4	31.1
DES2	ChCl	LacA	-	Water	1:3:0.97	6.4	26.1
DES3	ChCl	LacA	-	Water	1:4:0.99	7.1	21.3
DES4	ChCl	LacA	-	Water	1:5:0.98	7.5	18.9
DES5	ChCl	LacA	1,3-propanediol	Water	1:1:1:0.92	3.4	25.5
DES6	ChCl	LacA	1,3-propanediol	Water	1:2:1:0.95	4.8	18.2
DES7	ChCl	LacA	1,3-propanediol	Water	1:3:1:0.91	5.6	15.9
DES8	ChCl	LacA	1,3-propanediol	Water	1:4:1:0.92	6.4	15.3
DES9	ChCl	LacA	1,3-propanediol	Water	1:5:1:0.91	6.8	14.9
DES10	ChCl	LacA	1,3-butanediol	Water	1:1:1:0.93	2.9	30.0
DES11	ChCl	LacA	1,3-butanediol	Water	1:2:1:0.92	4.5	22.9
DES12	ChCl	LacA	1,3-butanediol	Water	1:3:1:1	5.4	18.6
DES13	ChCl	LacA	1,3-butanediol	Water	1:4:1:1	6.1	16.7
DES14	ChCl	LacA	1,3-butanediol	Water	1:5:1:1	6.6	17.7
DES15	ChCl	LacA	1,4-butanediol	Water	1:1:1:0.96	3.0	30.1
DES16	ChCl	LacA	1,4-butanediol	Water	1:2:1:0.92	4.5	21.2
DES17	ChCl	LacA	1,4-butanediol	Water	1:3:1:0.92	5.5	18.8
DES18	ChCl	LacA	1,4-butanediol	Water	1:4:1:0.91	6.2	15.2
DES19	ChCl	LacA	1,4-butanediol	Water	1:5:1:0.91	6.7	14.4
DES20	ChCl	LacA	1,5-pentanediol	Water	1:1:1:0.87	3.9	29.8
DES21	ChCl	LacA	1,5-pentanediol	Water	1:2:1:0.98	5.2	22.3
DES22	ChCl	LacA	1,5-pentanediol	Water	1:3:1:0.90	5.9	19.5
DES23	ChCl	LacA	1,5-pentanediol	Water	1:4:1:0.90	6.7	18.0
DES24	ChCl	LacA	1,5-pentanediol	Water	1:5:1:0.96	6.9	15.1

Since all extractions were performed at 60 °C, the viscosity values at just 60 °C are given here. Complete viscosity data are gathered in Table 4.

Table 3. Characterization of different properties of DES (conductivity, refractive index, and density).

	Conductivity (mS/cm)	Refractive Index	Density (g/cm^3)					
	25 °C	25 °C	25 °C	35 °C	45 °C	55 °C	65 °C	75 °C
DES1	1.87	1.4647	1.197	1.197	1.197	1.197	1.196	1.193
DES2	1.84	1.4562	1.099	1.099	1.099	1.099	1.099	1.099
DES3	1.76	1.4523	1.094	1.094	1.094	1.094	1.094	1.094
DES4	1.70	1.4499	1.070	1.070	1.070	1.070	1.069	1.068
DES5	3.45	1.4700	1.099	1.098	1.098	1.098	1.098	1.098
DES6	3.30	1.4614	1.078	1.078	1.078	1.078	1.078	1.077
DES7	2.99	1.4553	1.076	1.076	1.076	1.076	1.076	1.075
DES8	2.60	1.4516	1.063	1.063	1.063	1.063	1.063	1.062
DES9	2.28	1.4488	1.051	1.051	1.051	1.051	1.051	1.051
DES10	2.01	1.4689	1.083	1.083	1.082	1.082	1.082	1.082
DES11	1.95	1.4605	1.079	1.079	1.079	1.079	1.078	1.077
DES12	1.93	1.4547	1.073	1.073	1.073	1.073	1.073	1.073
DES13	1.76	1.4515	1.037	1.036	1.036	1.036	1.036	1.035
DES14	1.59	1.4484	1.029	1.028	1.028	1.028	1.028	1.027
DES15	2.44	1.4703	1.068	1.068	1.068	1.068	1.068	1.068
DES16	2.38	1.4619	1.067	1.067	1.067	1.067	1.067	1.067
DES17	2.27	1.4559	1.056	1.056	1.056	1.055	1.055	1.055
DES18	2.20	1.4527	1.053	1.053	1.053	1.053	1.053	1.051
DES19	2.08	1.4499	1.017	1.017	1.017	1.017	1.017	1.017
DES20	2.24	1.4689	1.080	1.080	1.080	1.080	1.080	1.079
DES21	2.14	1.4541	1.060	1.060	1.060	1.060	1.059	1.059
DES22	2.10	1.4539	1.058	1.058	1.058	1.058	1.057	1.057
DES23	1.96	1.4506	1.044	1.044	1.044	1.044	1.044	1.043
DES24	1.81	1.4500	1.037	1.037	1.037	1.037	1.037	1.036

Values of conductivity, refractive index and density were determined as described elsewhere [26,28].

2.3. Plant Materials

Spruce bark (*Picea abies*) as an industrial waste was provided by the timber company Bioenergo Ltd. (Ruzomberok, Slovakia). The spruce bark was air dried at ambient temperature until constant weight, homogenized by grinding using a knife mill with a motor power of 7.5 kW and separated using sieves into fractions. The 1.0–1.4mm fraction of spruce bark was extracted using DESs and analyzed to determine the content of holocellulose (52.0% ± 0.2%), lignin (26.4% ± 1.3%), ash (3.6% ± 0.4%) and extractives (12.7% ± 0.01%). The humidity of the material (8.77% ± 0.08%) was determined by drying approximately 1g of spruce bark at 105°C for 6 hours until complete water removal.

2.4. Extraction

The extraction conditions were similar to those described in Škulcová et al. [29]. Homogeneous samples of bark were withdrawn from the bark storage system in all extraction experiments. The dried and weighed ground bark was added to the DESs at a 1:20 (wt/wt) ratio. The extraction was performed for 2 h at 60 °C under continuous stirring in a closed flask.

When deciding on the application of DESs on an industrial scale, one of the key factors is their thermal stability. This stability is not just a function of thermal stability of their constituents but is also influenced by hydrogen bonds and electrostatic interactions, both decreasing with increases in temperature. High temperature might cause changes in the mass of DES due to its evaporation or decomposition. Haz et al. [62] investigated long-term isothermal stability of DESs (10 hours, 60–120 °C) composed of choline chloride and an organic acid (lactic, tartaric or malonic). Based on the results obtained it may be said that DESs investigated are stable at 60 °C. Lynam et al. [63] investigated in detail five DESs containing an organic acid (lactic, formic, acetic) and an amino acid (betaine, proline) or chloride choline. The thermal analysis took place in an inert nitrogen atmosphere at a heating rate 20 °C/min in the temperature range 30–100 °C, subsequently followed by heating rate 10 °C/min in 100–160 °C. The selected temperature limit 160 °C represented the maximum temperature of biomass processing. The authors compared boiling temperatures of the DESs respective constituents and thermal stability of the DESs. All DESs investigated were thermally stable up to 160 °C [63]. Based on the results above, it can be concluded that all the DESs used are thermally stable. The water content in the DESs was determined by coulometric Karl-Fischer titration, and during extraction in a closed flask it did not change.

2.5. Determination of Total Phenolic Content

The TPC in the extracts was estimated spectrometrically according to our previous work [29], based on redox reactions of Folin-Ciocalteu's reagent with phenols. First, 0.25 g of the extract was added into a 10 mL flask, and the flask was filled with ethanol. A total of 0.25 mL from the stock solution was mixed with 0.25 mL of Folin-Ciocalteu's reagent and 1.25 mL of 20% Na_2CO_3 p.a. solution in a 10 mL volumetric bank, which was then filled with distilled water. After agitation and standing for 1 h at an ambient temperature, the absorbance of the solution was measured against blanks in 0.5 cm cells at a wavelength of 765 nm. The phenolic compounds were expressed as gallic acid equivalent (GAE) in 100 g of extract using a calibration curve in the form of a straight line. All measurements were performed three times for each individual sample. The data in Table 5 represent average values; the differences in measurements did not exceed 3%.

2.6. Determination of Antioxidant Activity

The antioxidant activity was determined as free radical scavenging activity (RSA), using a standard method [64] based on the discoloration of the samples after reacting with the stable free 2,2-diphenyl-1-picrylhydrazyl radical (•DPPH), and subsequent absorbance measurements at 517 nm. Briefly, 3.5 mg/mL of extract was mixed with fresh •DPPH (0.08 mg/mL in methanol) solution at a ratio of 1:1 (vol/vol). The absorbance of the tested extracts, measured at 517 nm, was read against a blank

(methanol) after 0, 5, 10, 15, 20, 25 and 30 min. Gallic acid was used as a reference and corresponded to 100% activity. The RSA was calculated using Equation (1):

$$\text{RSA (\%)} = 100 \times (A_0 - A_{TEST})/(A_0 - A_{REF}) \quad (1)$$

where A_0 is the initial absorbance of the •DPPH solution in methanol, A_{TEST} is the absorbance of the tested sample in the •DPPH solution, and A_{REF} is the absorbance of gallic acid (0.7 mg/mL in methanol) in the •DPPH solution.

2.7. Determination of Viscosity

The viscoelastic properties were evaluated using a Brookfield DVII + Pro viscometer, as described earlier [27,28]. The sample viscosity was measured at different temperatures (30–90 °C) and revolutions (5, 10, 20 and 50 rpm), using a spindle 18 with an adapter. All the measurements were performed three times on individual samples. The error of measurement for individual viscosities is in the range of 0.2 to 0.5 mPa·s. Temperature and rpm dependences of viscosity for the used DESs are listed in Table 4. The resulting viscosity for different temperatures is expressed as the average value for different revolutions. All systems behaved as Newtonian liquids.

Table 4. Viscosity of the studied DESs for different temperatures and revolutions (5–50 rpm).

DES1: ChCl:LacA:Water (1:2:0.96)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	133.8	134.4	134.1	x	134.1
40	79.2	78.6	78.1	x	78.6
50	49.2	47.4	47.2	48.1	48.0
60	30.0	31.5	31.6	31.3	31.1
70	22.2	22.8	22.3	22.3	22.4
80	16.2	16.5	16.8	16.6	16.5
90	13.2	12.9	13.8	13.6	13.4
DES2: ChCl:LacA:Water (1:3:0.97)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	94.8	96.0	96.7	x	95.8
40	58.2	57.6	56.7	57.2	57.4
50	36.0	35.1	35.7	35.5	35.6
60	25.2	26.4	26.2	26.5	26.1
70	17.4	18.9	18.6	18.4	18.3
80	15.0	13.8	13.9	14.1	14.2
90	11.4	11.4	10.8	10.6	11.1
DES3: ChCl:LacA:Water (1:4:0.99)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	78.6	79.2	79.2	x	79.0
40	49.8	47.7	47.2	48.0	48.2
50	29.0	30.9	30.7	30.3	30.2
60	21.6	21.6	21.0	21.1	21.3
70	16.8	15.0	15.6	15.4	15.7
80	12.6	12.6	12.6	12.6	12.6
90	9.6	9.6	9.9	10.0	9.8

Table 4. *Cont.*

DES4: ChCl:LacA:Water (1:5:0.98)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	73.2	71.7	71.4	x	72.1
40	42.6	41.7	42.1	42.5	42.2
50	26.4	27.3	27.1	27.1	27.0
60	19.0	19.2	18.6	18.8	18.9
70	12.6	14.2	14.2	13.7	13.7
80	11.1	11.4	11.1	11.1	11.2
90	8.4	8.7	8.9	9.1	8.8
DES5: ChCl:LacA:1.3-propanediol:Water (1:1:1:0.92)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	86.4	85.5	84.9	x	85.6
40	57.2	55.2	54.4	54.3	55.3
50	36.6	36.0	36.4	36.0	36.3
60	25.2	26.4	25.5	25.0	25.5
70	18.6	18.9	18.1	18.1	18.4
80	14.4	13.8	13.8	13.9	14.0
90	10.8	10.5	10.6	10.7	10.7
DES6: ChCl:LacA:1.3-propanediol:Water (1:2:1:0.95)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	65.4	64.2	63.6	x	64.4
40	40.2	38.7	40.0	40.1	39.8
50	26.4	27.0	25.8	26.2	26.4
60	18.6	17.7	18.3	18.1	18.2
70	13.2	12.9	13.5	13.7	13.3
80	10.8	10.2	10.2	10.3	10.4
90	8.2	8.1	8.4	8.4	8.3
DES7: ChCl:LacA:1.3-propanediol:Water (1:3:1:0.91)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	57.6	57.6	56.4	56.4	57.0
40	34.8	33.6	34.9	34.8	34.5
50	21.0	23.1	22.6	22.8	22.4
60	15.6	16.2	16.0	15.8	15.9
70	12.6	11.7	11.8	12.0	12.0
80	9.0	9.3	9.1	9.1	9.1
90	7.2	7.8	7.6	7.6	7.6
DES8: ChCl:LacA:1.3-propanediol:Water (1:4:1:0.92)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	51.6	50.1	49.8	50.5	50.5
40	31.2	31.2	31.3	31.0	31.2
50	21.6	22.2	21.7	22.1	21.9
60	15.3	15.0	15.6	15.4	15.3
70	12.6	11.4	11.7	11.6	11.8
80	8.4	9.0	9.0	9.1	8.9
90	6.6	7.5	7.4	7.3	7.2

Table 4. *Cont.*

DES9: ChCl:LacA:1.3-propanediol:Water (1:5:1:0.91)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	46.2	44.4	44.2	44.6	44.9
40	28.8	28.2	28.2	27.9	28.3
50	19.8	21.6	20.4	20.2	20.5
60	15.6	14.4	14.8	14.7	14.9
70	12.0	11.4	11.1	10.7	11.3
80	11.4	10.8	10.8	10.5	10.9
90	9.6	9.6	8.9	8.8	9.2
DES10: ChCl:LacA:1.3-butanediol:Water (1:1:1:0.93)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	118.2	117.9	118.5	x	x
40	72.0	71.4	71.1	x	x
50	46.8	45.3	44.7	45.4	45.4
60	29.4	29.7	30.4	30.0	30.0
70	21.6	21.9	20.8	21.1	21.1
80	16.2	15.6	15.9	15.8	15.8
90	12.6	12.6	12.1	12.1	12.1
DES11: ChCl:LacA:1.3-butanediol:Water (1:2:1:0.92)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	86.4	86.1	85.8	x	86.1
40	52.8	51.9	51.1	51.9	51.9
50	33.0	32.7	33.1	32.8	32.9
60	22.8	22.5	21.9	22.2	22.4
70	16.2	15.6	16.0	15.7	15.9
80	12.0	12.0	11.8	11.9	11.9
90	9.6	10.5	9.9	10.1	10.0
DES12: ChCl:LacA:1.3-butanediol:Water (1:3:1:1)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	72.6	71.7	71.4	x	71.9
40	43.2	42.0	42.7	42.8	42.7
50	25.8	27.3	27.4	27.2	26.9
60	19.2	18.6	18.1	18.3	18.6
70	13.8	12.3	13.5	13.3	13.2
80	10.2	10.5	10.2	10.3	10.3
90	7.8	9.0	8.8	9.0	8.7
DES13: ChCl:LacA:1.3-butanediol:Water (1:4:1:1)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	63.6	63.0	62.4	x	63.0
40	38.4	38.1	38.5	38.5	38.4
50	23.4	24.9	24.3	24.5	24.3
60	17.4	16.2	16.8	16.5	16.7
70	12.0	12.3	12.1	12.1	12.1
80	9.6	9.6	9.4	9.4	9.5
90	6.6	7.2	7.1	7.4	7.1

Table 4. *Cont.*

DES14: ChCl:LacA:1.3-butanediol:Water (1:5:1:1)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	63.6	60.9	60	59.9	61.1
40	38.4	36.7	36.3	35.9	36.8
50	24.6	26.1	24.0	23.7	24.6
60	19.8	17.7	16.8	16.6	17.7
70	13.8	12.6	12.7	12.3	12.9
80	10.2	10.5	9.0	9.1	9.7
90	8.4	8.4	8.4	8.5	8.4
DES15: ChCl:LacA:1.4-butanediol:Water (1:1:1:0.96)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	107.4	105.9	106.9	x	106.7
40	69.6	68.7	68.2	x	68.8
50	45.0	43.2	43.8	44.1	44.0
60	30.0	30.3	30.1	30.0	30.1
70	21.6	21.9	21.1	21.3	21.5
80	16.0	15.6	16.0	15.9	15.9
90	12.0	12.9	11.8	11.9	12.2
DES16: ChCl:LacA:1.4-butanediol:Water (1:2:1:0.92)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	77.4	77.1	76.8	x	77.1
40	48.0	46.5	46.5	47.1	47.0
50	30.6	30.0	30.6	30.5	30.4
60	21.3	21.3	21.0	21.1	21.2
70	16.2	14.7	15.4	15.2	15.4
80	13.2	12.0	12.3	12.2	12.4
90	9.0	9.9	9.3	9.7	9.5
DES17: ChCl:LacA:1.4-butanediol:Water (1:3:1:0.92)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	64.8	63.3	62.7	x	63.6
40	39.6	38.4	39.7	39.7	39.4
50	25.2	27.3	26.1	26.5	26.3
60	19.2	18.9	18.7	18.2	18.8
70	14.4	14.1	14.4	14.2	14.3
80	10.8	11.1	11.4	11.3	11.2
90	9.6	9.6	9.5	9.5	9.6
DES18: ChCl:LacA:1.4-butanediol:Water (1:4:1:0.91)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	57.0	56.1	54.6	55.2	55.7
40	34.2	33.0	34.0	33.7	33.7
50	21.6	23.1	22.0	22.1	22.2
60	15.0	15.0	15.6	15.3	15.2
70	12.6	12.0	11.8	11.5	12.0
80	9.6	9.9	9.0	9.2	9.4
90	7.5	7.5	7.4	7.9	7.6

Table 4. *Cont.*

DES19: ChCl:LacA:1.4-butanediol:Water (1:5:1:0.91)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	52.8	50.7	50.1	50.5	51.0
40	31.2	31.5	31.5	31.3	31.4
50	21.0	21.3	20.2	20.3	20.7
60	14.4	14.4	14.5	14.2	14.4
70	10.8	10.5	10.4	10.3	10.5
80	7.2	8.1	8.1	8.2	7.9
90	6.6	6.6	6.5	6.7	6.6
DES20: ChCl:LacA:1.5-pentanediol:Water (1:1:1:0.87)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	107.4	108.6	109.3	x	108.4
40	69.0	68.7	68.4	x	68.7
50	45.0	43.0	43.9	44.3	44.1
60	28.8	30.0	30.4	30.1	29.8
70	21.0	21.6	21.3	21.5	21.4
80	16.2	15.9	16.2	16.0	16.1
90	12.6	12.3	12.6	12.4	12.5
DES21: ChCl:LacA:1.5-pentanediol:Water (1:2:1:0.98)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	81.6	79.2	79.3	x	80.0
40	51.6	48.6	48.3	49.1	49.4
50	31.2	31.5	31.8	31.7	31.6
60	21.6	22.8	22.2	22.4	22.3
70	15.0	15.5	16.5	15.9	15.7
80	12.0	12.3	12.4	12.2	12.2
90	9.0	10.2	9.3	9.7	9.6
DES22: ChCl:LacA:1.5-pentanediol:Water (1:3:1:0.90)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	76.2	74.7	74.2	x	75.0
40	47.4	44.7	44.5	44.9	45.4
50	28.8	29.4	29.1	28.7	29.0
60	19.2	19.8	19.3	19.5	19.5
70	14.4	13.8	14.5	14.1	14.2
80	10.8	11.1	10.6	10.5	10.8
90	7.8	8.7	7.9	8.3	8.2
DES23: ChCl:LacA:1.5-pentanediol:Water (1:4:1:0.90)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	61.8	61.8	61.6	x	61.7
40	37.2	37.2	37.9	37.9	37.6
50	25.2	26.1	25.6	25.9	25.7
60	18.6	17.7	18.0	17.7	18.0
70	13.2	13.2	12.9	12.9	13.1
80	10.8	10.5	9.8	9.8	10.2
90	8.4	8.4	8.4	8.4	8.4

Table 4. *Cont.*

DES24: ChCl:LacA:1.5-pentanediol:Water (1:5:1:0.96)					
Temperature (°C)	**Viscosity (mPa s)**				**Average Viscosity (mPa·s)**
	5 rpm	**10 rpm**	**20 rpm**	**50 rpm**	
30	55.8	54.9	53.8	54.9	54.9
40	32.7	32.7	33.6	33.3	33.1
50	21.6	22.5	21.4	21.8	21.8
60	15.0	14.8	15.2	15.4	15.1
70	10.8	11.1	11.2	11.2	11.1
80	7.8	9.0	8.8	9.0	8.7
90	6.6	6.9	7.1	7.0	6.9

3. Results and Discussion

The extraction of phenolic compounds using DES systems has been performed and the results have been evaluated. Water is the most common solvent. It is able to form hydrogen bonds and plays the role of both hydrogen bond donor and acceptor [65,66]. Addition of water into DESs in the process of their formation causes incorporation of water molecules into the structure of DESs, their fixation by hydrogen bonds, and this water cannot be later fully removed by, e.g., a rotary evaporator. Different content of water in the studied DESs indicates that water molecules bind predominantly to hydrogen bond donors [66]. A small addition of water may result in a decrease in viscosity, temperature lowering and shorter time needed for DES preparation. Moreover, the presence of water may change the ability to dissolve some compounds in DES, e.g., plant metabolites [16]. Water influences the course of reactions where a DES acts as a catalyst promoter. Understanding how water activity changes the DESs properties is key for researchers looking to encourage or retard biological growth in addition to studies of proton-coupled electron transfer, solvation, or any other water-coupled or water-dependent process [67]. Smith et al. [67] examined the influence of water in the system of choline chloride and urea (1:2) which was published in a paper in 2019. Based on their results it is possible to draw a conclusion that if DES systems contain water, it is necessary to take water into account as another component of the DES. Therefore, binary systems need to be characterized as ternary.

In this work, water containing choline chloride-based deep eutectic solvents (DESs) with lactic acid and 1,3-propanediol, 1,3-butanediol, 1,4-butanediol, and 1,5-pentanediol, with different molar ratios (Table 2), were used as extractants. Twenty-four different DESs (Table 1) were applied as extractants. As shown in Table 5, the content of polyphenols in the eutectic extracts ranged from 177.6 to 596.2 mg of gallic acid equivalent (GAE) per 100 g of dry bark.

The amount of total phenolic compounds varied widely in different plant materials (92 phenolic extracts from acetone and methanol were examined) and ranged from 0.2 to 155.3 mg GAE/g dry material [68]. The yields of phenolic compounds were generally variable depending on the tree species, part of the tree used for extraction, as well as the age or place of origin of the tree [69,70]. According to the literature, in forest pine, the total amount of polyphenols ranges between 76 mg GAE /g in dry bark, 17.5 mg GAE/g in needles, and 1.1 mg GAE/g in cork wood [48]. The concentration of phenolic compounds in spruce knotwood is 10–15%, and even up to 30%, of absolute dry weight, while in pine knotwood it is less than 10% dry weight, with concentrations several times lower than that observed for logs [71]. According to [71], more than half of the hydrophilic extractive substances in lignite are found in knotwood.

In the previous paragraph, the extractions using organic solvents are presented. The next paragraph deals with our experiments applying DESs.

Table 5. Total phenolic contents of the spruce bark extracts obtained with DESs.

Sample	TPC (mg GAE/100 g Extract)	TPC (mg GAE/100 g Dry Bark)
DES1	15.7 ± 0.1	393.6 ± 3.0
DES2	13.9 ± 0.3	336.9 ± 6.5
DES3	12.8 ± 0.2	326.7 ± 3.0
DES4	13.4 ± 0.1	349.4 ± 2.6
DES5	11.6 ± 0.2	288.1 ± 2.5
DES6	13.8 ± 0.4	336.5 ± 8.7
DES7	12.0 ± 0.3	312.3 ± 7.3
DES8	14.0 ± 0.1	361.6 ± 2.2
DES9	13.8 ± 0.1	343.8 ± 2.1
DES10	9.4 ± 0.1	233.6 ± 1.8
DES11	10.9 ± 0.4	287.6 ± 8.5
DES12	11.6 ± 0.1	277.6 ± 2.1
DES13	20.9 ± 0.2	531.4 ± 3.9
DES14	23.4 ± 0.3	596.2 ± 7.4
DES15	12.1 ± 0.1	283.6 ± 2.0
DES16	13.4 ± 0.1	331.7 ± 2.5
DES17	12.3 ± 0.3	313.3 ± 7.4
DES18	13.7 ± 0.2	337.9 ± 3.9
DES19	12.6 ± 0.1	339.1 ± 1.8
DES20	13.7 ± 0.1	332.6 ± 2.1
DES21	14.3 ± 0.1	350.4 ± 3.0
DES22	14.6 ± 0.2	363.9 ± 2.8
DES23	11.3 ± 0.2	291.8 ± 5.1
DES24	16.0 ± 0.1	422.4 ± 2.4

A series of experiments applying DESs was performed to examine the effect of lactic acid as a hydrogen bond donor on the efficiency of polyphenol extraction. In the series DES1 to DES4, the choline chloride: lactic acid: water molar ratio was varied from 1:2:0.96 to 1:5:0.98. The results showed that the highest polyphenol content was measured for the extract with the 1:2:0.96 molar ratio (396.6 mg GAE/100 g dry bark). Thus, increasing the HBD (lactic acid) content in the extractant did not cause an increment in the TPC. In contrast, at the molar ratio of 1:3:0.97, the polyphenol content was 336.9 mg GAE/100 g dry bark, and at 1:4:0.99 – only 326.7 mg GAE/100 g dry bark. At the molar ratio of 1:5:0.98, the TPC increased to 349.4 mg GAE/100 g dry bark.

It is known that the recovery of polar compounds from samples can be enhanced varying the extraction conditions (diffusivity, density, viscosity) and with the addition of a co-solvent [53]. As shown in Table 1, viscosity decreases with increasing HBD content. Thus, we assumed that, under the same extraction conditions, the TPC should increase with a decrease in viscosity. However, this has not been confirmed.

As mentioned earlier, the addition of a co-solvent also affects the amount of substances obtained. The addition of a co-solvent (e.g., ethanol) is intended to significantly swell plant cells, allowing better solvent penetration and diffusion of the solute present in the solid lignocellulosic matrix [72]. Similar behavior has been demonstrated in several previous studies [73–75]. However, the use of a higher concentration of the co-solvent can also reduce the yield of bioactive compounds because of CO_2 -co-solvent interactions [59].

In our case, we added another HBD to the system, namely 1,3-propanediol, 1,3-butanediol, 1,4-butanediol, or 1,5-pentanediol. The aim was to reduce the viscosity and thus improve the extraction process. As a result, the viscosity of these systems was reduced at 60 °C, and therefore we assumed that the TPC would increase when applying these extraction systems. We found that in the DES5 to DES9 systems (i.e., ChCl : LacA : 1,3-propanediol : water (molar ratios 1 : 1 : 1: 0.92; 1 : 2 : 1 : 0.95; 1 : 3 : 1 : 0.91; 1 : 4 : 1 : 0.92; 1 : 5 : 1 : 0.91), the TPC reached 288.1; 336.5; 312.3; 361.3 and 343.8 mg

GAE/100 g dry bark, respectively. Thus, the addition of 1,3-propanediol had no significant effect on the TPC and, in some cases, there was even a decrease in the TPC. In the case of the system containing 1,3-butanediol, the TPC ranged from 277.6 to 562.2 mg GAE/100 g dry bark. When using 1,4-butanediol, the TPC reached 283.6–337.9 mg GAE/100 g dry bark. Thus, the changes in the extraction system and viscosity (viscosity at 60 °C ranged from 30.1 mPa s to 14.4 mPa s) altered the TPC. The addition of 1,5-pentanediol resulted in TPC ranging from 291.8 to 422.4 mg GAE/100 g dry bark. The highest TPC was achieved by the system having a 1 : 5 : 1 : 0.96 molar ratio (422.4 mg GAE/100 g dry bark). For the 1 : 3 : 1 : 0.9 system, the TPC increased by 8% over the ChCl : LacA : Water system (1 : 3 : 0.97). For other extraction systems, a decrease in the TPC was achieved, despite the reduced viscosity, compared to the diol-free systems (DES1, DES3).

Based on this evaluation of the investigated 24 extraction systems, we can conclude that the system containing ChCl : LacA : 1,3-butanediol : water (1 : 4 : 1 : 1; 1 : 5 : 1 : 1) achieved the best results in polyphenols extraction. Both systems displayed a significant increase in the TPC for the 1 : 4 : 1 : 1 system, the TPC was 531.4 mg GAE/100 g dry bark, while for the 1 : 5 : 1 : 1 molar ratio, the TPC was up to 596.2 mg GAE/100 g dry bark (23.4 mg GAE/100 g extract).

As mentioned in the Introduction, the extraction of polyphenols from various wastes is a very important area of research. Nevertheless, to the best of the authors' knowledge, only one paper has been published on the extraction of spruce bark using DESs [29]. Using 41 DESs, the polyphenols content was reported to range from 41 to 463 mg GAE/100 g of extract (or 9 to 100 mg GAE per 1 g dry weight [29]).

In this work, the TPC content achieved in the extract ranged from 9.4 to 23.4 mg GAE/100 g extract (177.6 to 596.2 mg GAE/100 g of dry bark). From the viewpoint of implementing DESs in practice, it is necessary to compare the TPC reached using DESs with that achieved using common organic solvents (taking into account the impact of bark diversity). Working with ethanol-water mixtures (50%–70% v/v ethanol) at 40–60 °C and extraction time of 30–60 minutes, applying UAE, the TPC was in the range of 6.62–13.32 mg GAE/g spruce bark [10]. The TPC in the spruce bark extract (*Picea abies* L.) using the classical method (water batch extraction) was 113.56 mg GAE/g extract, while when the sonication method was used, the TPC value was lower (84.28 mg GAE/g extract) [76]. Spinelli et al. [7], published the results obtained using supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), and ultrasound-assisted extraction (UAE) of Norway spruce bark by ethanol. The TPC for SFE and different ethanol concentrations were as follows: 10% ethanol: 0.77 mg GAE/g DM; 20% ethanol: 1.24 mg GAE/g DM; and 40% ethanol: 2.5 mg GAE/g DM. For PLE and water, 33.45 mg GAE/g DM was determined; for 98% ethanol, 46.34 mg GAE/g DM was measured. Using UAE and 98% ethanol, 54.97 mg GAE/g DM was found.

In addition to the solvent type, the polyphenol content also depends largely on the bark particle size. Sládkova et al. [77] found that using MAE, the TPC varied between 42.7 and 265.0 mg GAE per 100 g of dry bark for different particle sizes (0.3; 1 and 2.5 mm) at an extraction temperature of 60 °C. When the particle size was 1 mm, the TPC ranged from 90.3 to 321.1 mg GAE/100 g dry bark. It is known that, normally, the amount of extracts will depend on many factors (particle size, method used, extracting agent, extraction conditions, and others) and a simple comparison is thus inadequate, being rather indicative. In this investigation, it has been shown that by changing the reagent through the addition of a third component, in some cases, it is possible to obtain a higher TPC. This is particularly related to a change in the polarity of the extraction system due to the addition of diols. In our work, the extraction system was very simple, a DES was mixed with the spruce bark at 60 °C and the mixture was extracted for 2 hours. As suggested by other authors regarding the treatment of various kinds of biomass using MAE and UAE, the yield of extracted polyphenols can be higher than that using conventional reagents [78–81].

A separate part of the work focused on the determination of the antioxidant capacity of the extracts. Radical scavenging activity of phenolic compounds is stemming from their ability to act as reducing agents, hydrogen or electrons donors, and singlet oxygen quenchers [82]. Table 6 summarizes

the antioxidant activity measured at 0–30 min after the addition of •DPPH. The differences in radical scavenging activity (RSA) suggested that each DES preferentially dissolved another type of extractive with a differing reactivity to •DPPH. Moreover, each DES had a different extraction of TPC, and thus, the amount of extractives had an impact on the antioxidant activity and on the reaction with the radical. To clear up the matter, extractives are individual compounds reacting with •DPPH. RSA values relate to extracts (DES components and extractives) and were found to vary from 81.4% to 95%. A lower antioxidant activity was observed for the extracts obtained with ChCl : LacA : Water (1 :2 : 0.96), RSA 86.4%, and for the system containing ChCl : LacA and different diols in a molar ratio of 1 : 1 : 1, namely 82.4% for 1,3-propanediol; 84.2% for 1,3-butanediol; 85.4% for 1,4-butanediol, and 81.4% for 1,5-pentanediol. The ChCl : LacA : 1,3-butanediol : Water (1 : 5 : 1 : 1) extracts had the highest antioxidant activity, with RSA of 95% and this extract also contained the highest content of polyphenols (596.2 mg GAE/100 g dry bark).

In several works previously reported by other authors, focus is laid on finding a correlation between TPC and antioxidant activity [29,40,82,83]. In our study, we also tried to find such a correlation, however, with no success (figure not shown), the correlation coefficient (R^2) was just 0.12.

Table 6. 2,2-diphenyl-1-picrylhydrazyl radical (•DPPH) assay of antioxidant activity (RSA) for different extracts.

Sample	RSA (%)						
Time (min)	**0**	**5**	**10**	**15**	**20**	**25**	**30**
DES1	76.2	78.6	81.0	82.7	84.3	85.4	86.4
DES2	83.3	86.2	88.2	89.8	90.9	92.2	93.2
DES3	85.9	88.0	89.5	90.8	91.7	92.5	93.2
DES4	83.7	86.2	88.1	89.6	90.6	91.5	92.3
DES5	72.0	74.6	76.8	78.5	79.9	81.3	82.4
DES6	81.4	84.3	86.2	87.9	89.3	90.4	91.3
DES7	85.2	87.9	89.7	91.3	91.7	93.1	93.8
DES8	86.2	88.7	90.6	91.9	92.8	93.4	94.1
DES9	86.6	89.4	91.1	92.5	93.4	94.0	94.6
DES10	69.0	74.7	78.0	80.2	81.8	83.1	84.2
DES11	70.6	76.6	80.0	82.3	84.3	85.6	86.9
DES12	74.7	81.7	85.0	87.6	89.4	90.5	91.7
DES13	75.7	82.7	86.4	89.0	90.6	91.8	92.6
DES14	80.8	87.9	91.0	92.8	93.8	94.5	95.0
DES15	70.0	75.5	78.8	81.1	82.8	84.3	85.4
DES16	76.6	82.9	86.3	88.5	90.2	91.3	92.3
DES17	76.0	82.5	86.0	88.3	89.8	91.1	92.1
DES18	78.7	85.1	88.5	90.6	92.1	93.1	93.8
DES19	77.1	84.3	87.5	91.2	92.8	93.4	94.1
DES20	71.5	74.0	75.7	77.5	78.9	80.1	81.4
DES21	78.9	81.8	83.8	85.4	86.9	88.0	88.8
DES22	79.4	82.2	84.3	86.0	87.5	88.5	89.7
DES23	82.6	89.0	92.0	93.4	94.2	94.7	94.9
DES24	74.3	80.1	83.5	85.8	87.5	88.8	89.8

The recovery of polyphenolic substances from agri-food and forestry wastes is an important target in the future of biorefining and numerous studies have been oriented towards the valorization of processing wastes by the exploitation of side streams, implementing eco-friendly and cost-effective technologies [1–3,13,16–18,79–81,84–90].

The zero-waste biorefinery concept was supported by the work of Li et al. [87], who studied 12 refined oils, including grapeseed, rapeseed, peanut, sunflower, olive, avocado, almond, apricot, corn, wheat germ, soybean, and hazelnut oils to extract substances from rosemary leaves. The zero-waste

concept deserves attention from the perspective of using DESs as well. One of the aims of using DESs may be to obtain an enriched solvent, without requiring any further process to separate the extracted substances. This enriched solvent would be used as syrup, either as a nutritional supplement with antioxidants, suitable for direct consumption, or as a preparation for skin, hair, and so on. treatment. Thus, this extraction technology would reduce the amount of waste generated, but would also be a cost-effective method for the isolation of value-added substances from forestry or agri-food residues. Moreover, the recycling process would be unnecessary, since the waste resulting after the extraction, especially in the case of agri-food waste, could be further used as animal feed by selecting the appropriate type of solvents for the extraction.

As far as the properties of choline chloride are concerned, it is characterized as a harmless compound, furthermore, it is an essential nutrient [82] and is used in the treatment of several diseases. As far as the price of choline chloride is concerned, high-quality 99% food grade or 99% pharmaceutical grade choline chloride is supplied for 10–30 $/kg; industrial 98% choline chloride is sold for 5–9 $/kg at minimal order of 20 kg (data available from www.alibaba.com). It thus can be considered as a cheap chemical. Similarly, lactic acid is low-cost, its price being of 1.1–2 $/kg, while that of ethanol is 0.5–1.5 $/kg.

When advocating the practical advantages of DESs, one of the most important supporting arguments is their ability of recuperation and multiple use [13]. Generally, DES recycling lies in the use of an anti-solvent, causing the elimination of a component from the system under operation, and after removal of water by evaporation, purified DES is obtained and may be reused [13].

On the other hand, it has been confirmed that, when certain types of DESs are applied, they achieve greater extractability of polyphenols than conventional solvents [78,89,90]. The antioxidant capacity achieved is also higher than that reached using conventional solvents [79–81]. It also appears that the extraction time at the same yield of extracted substances is lower when using DESs. In addition, it has been found that these solvents can be combined with other techniques, such as microwave-assisted or ultrasound-assisted extraction, which speed up the extraction time, extractive yield and, at the same time, use less solvent for the extraction [79–81].

4. Conclusions

This study focused on the application of deep eutectic solvents in spruce bark extraction. Twenty-four solvents based on combinations of choline chloride with lactic acid and 1,3-propanediol, 1,5-pentanediol, 1,4-butanediol, 1,3-butanediol, and water, with different molar ratios, were used as extractants under conditions of 120 min extraction time and a temperature of 60 °C. The total phenolic content and antioxidant activity of the extracts were determined. The results from the TPC analysis indicated that the extract achieved with choline chloride, lactic acid, 1,3-butanediol and water (1 : 5 : 1 : 1) had the highest antioxidant activity and radical scavenging activity of 95%. Also, this extract contained the highest content of polyphenols (596.2 mg GAE/100 g dry bark).

A comparison of the TPC and RSA values obtained in bark extraction using DESs under ambient conditions with those reached with organic solvents at higher temperature and pressure shows that the organic solvents are, in most cases, more effective. A comparison of the total cost associated with the use of the two mentioned classes of extractants may, however, lead to contrasting results. The reason lies in the cost of handling, cleaning and recovering organic solvents, the costs of generating and maintaining high temperature and pressure, as well as the costs of maintaining the quality of the living and working environment. It can be assumed that, over time, the factors mentioned will favor the use of DESs over that of organic solvents.

When evaluating the use of antioxidants extracted from biomass, two new aspects are emerging. The first is that, along with the traditional applications of antioxidants of natural origin in medicine, pharmacy, cosmetics, and food industry, they might be introduced into other fields as well. The following areas can be suggested as examples: the stabilization of dyes in the textile industry [91,92]; the stabilization of biofuels [93,94]; the stabilization of polymers [95,96]; and metalworking [97].

Of course, any application requires knowledge of the mechanism of action of antioxidants [98]. The second aspect is related to the form of use of antioxidants. It would be very advantageous if extracts could be used instead of isolated individual antioxidants. In this regard, the use of DESs for the extraction of antioxidants seems to be particularly convenient.

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Nomenclature

RSA—radical scavenging activity (%)TPC—total phenolic content (mg GAE/100 g dry bark)

Abbreviations

ABTS—2,20 -azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ASE—accelerated solvent extraction
ChCl—choline chloride
DES—deep eutectic solvent
DM—dry material
DPPH—2,2-diphenyl-1-picrylhydrazyl radical
FRAP—ferric reducing antioxidant power
GAE—gallic acid equivalents
HBA—hydrogen bond acceptor
HBD—hydrogen bond donor
LacA—lactic acid
MAE—microwave-assisted extraction
NADES—natural deep eutectic solvent
PLE—pressurized liquid extraction
SFE—supercritical fluid extraction
TEs—Trolox equivalent
UAE—ultrasound-assisted extraction

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