GREEN SOLVENTS BASED ON CHOLINE CHLORIDE FOR THE EXTRACTION OF SPRUCE BARK (*PICEA ABIES*)

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Spruce bark is a rich source of extractives, such as condensed tannins, suberin, resin acids and terpenes. Deep eutectic solvents (DESs), a new type of green solvents, were used in this study for obtaining a spruce bark extract with valuable properties. Choline chloride-based eutectic solvents with carboxylic acids and glycerol were used as extractants. The extractions were performed for 1 h at 60 °C under continuous stirring. The antioxidant activities were evaluated using an antioxidant system with 2,2-diphenyl-1-picrylhydrazyl (DPPH). The content of the total phenolics in the extracts was determined spectrometrically according to the Folin-Ciocalteu procedure and expressed as gallic acid equivalent (GAE). The results indicated promising possibilities for the development and usage of eutectic solvents for bark pretreatment. All the tested extracts showed phenolic contents that ranged from 41 to 463 mg GAE/100 g extract. No correlation between the total phenolic content and antioxidant activity was observed. This study demonstrated that DESs are environmentally suitable solvents for extracting phenolic compounds from spruce bark.

Keywords: extraction, spruce bark, deep eutectic solvent, choline chloride, antioxidant activity, total phenolic content

INTRODUCTION

Lignocellulose is the most abundant renewable biomass resource on earth, and it can be potentially used as a sustainable alternative for fuel and the production of chemicals.[1] Spruce bark is a natural source of celluloses, hemicelluloses, lignins, resins, tannins and terpenes. The valorisation of bark is a key factor in the usage of by-products from the lignocellulosic industry.[2] Forest biorefinery concepts aim to produce energy and reduce the cost of production of value-added products from bark. Several hundred million tons of bark are incinerated, landfilled, or used for thermal energy production annually without valorisation of the content. Bark is used as a cheap source of energy, while incineration and landfilling can lead to environmental problems. The combustion of bark produces a large amount of ash (more than wood), which damages combustors.[3] Softwood bark comprises a wide variety of antioxidants, of which phenolic antioxidants are of particular interest for fine chemical isolation and utilization. Typical phenolic antioxidants are flavonoids, phenolic acids, stilbenes, tannins, lignans and lignins.[2] A huge research and development effort is currently underway to develop sustainable processes to extract value-added products from bark. A promising technology is the use of deep eutectic solvents (DESs). DESs consist of two or more components and are referred to as green solvents. The melting point of a DES is lower than for any of its individual components.[4] DESs are composed of a hydrogen bond donor and a hydrogen bond acceptor. Eutectic mixtures have been used to fractionate biomass from wheat straw,[5,6] rice straw[7] and pine wood.[5] DESs can be used for a number of applications, such as the extraction of

flavonoids from plants,[8,9] solvents for organic synthesis,[10] chemical conversion,[11,12] enzymatic degradation,[14] dehydration,[11,15] or as solvents for extraction,[16-24]and medium for nanocrystal cellulose production.[25]

This research is the first report on the use of DESs as solvents for bark pretreatment. This study demonstrated that these environmentally friendly solvents may be treated as a new generation of extraction agents for industrial development. Even agents used for supercritical extraction, extraction under reflux and steam distillation, pressurized solvent extraction, or other conventional extraction processes can be replaced with DESs. Contrary to the previously mentioned techniques, DESs do not require temperatures above 100 °C or increased pressure during extraction. An interesting type of DESs is the natural deep eutectic solvent (NADES), which is composed of natural compounds isolated from insects, plants, animals, or microorganisms.[26] DESs are biodegradable, biocompatible and non-toxic, similar to NADESs and unlike organic solvents. The yields of extraction by organic solvents (petroleum ether, methylene chloride and n-hexane),[27-30] are between 1.8% and 12.0%, while the yields from DESs are between 11.40% and 27.7% (Table 3). DESs are becoming increasingly popular and have been applied to minimize environmental problems.[31] Choline chloride belongs to the group of B vitamins applied as a common supplement in poultry feed. Carboxylic acids can be recycled or degraded using advanced oxidation processes.[32] Bio-renewable natural compounds that have well-characterized biodegradable and toxicological properties are ideal materials from environmental and economic viewpoints.

In this study, DES was used to extract phenolic compounds from spruce (*Picea abies*) bark. This research evaluated the antioxidant activity of different extracts from spruce bark and the relationship between the total phenolic content (TPC) and antioxidant activity. Moreover, the possible relationship between the properties of the DESs and the extraction yield was investigated. This study may allow the purposeful utilization of DESs to achieve the desired quantity, composition and properties of the extractives.

EXPERIMENTAL

Materials

Spruce bark (Picea abies) characterisation

Spruce bark was obtained from Bioenergo Ltd. (Ruzomberok, Slovakia). The bark was ground (particle size <0.55 mm), sieved and dried.[33]Before extraction, the ground bark was dried and weighed. Samples were analyzed to determine the content of lignin, ash and holocellulose (Table 1). The lignin content was determined by the Klason lignin procedure,[34] and the ash was determined using TAPPI T413 om-11.[35] The holocellulose was quantified with the sodium chlorite treatment according to the procedure of Wise *et al*.[36]

Chemicals

Choline chloride was obtained from Sigma Aldrich, Bratislava, Slovakia (≥98%). Glycerol was purchased from Penta S.R.O., Slovakia (86%) (Table 2). Eight carboxylic acids were tested: tartaric acid (99.5%), lactic acid (90%, Sigma Aldrich), malonic acid (99%, Sigma Aldrich), malic acid (≥99%, Sigma Aldrich), maleic acid (≥99%, Sigma Aldrich), glycolic acid (99%, Sigma Aldrich), oxalic acid × 2H2O (≥99%, Sigma Aldrich), and citric acid × H2O (≥99%, Sigma Aldrich).

Methods

DES extraction

Choline chloride was mixed with carboxylic acid or glycerol. The mixtures were stirred in an oil bath to form a homogeneous liquid at 60 °C to 80 °C, depending on the carboxylic acid.

Table 1
Composition of spruce bark

Component	Content (%)
Holocellulose	51.67 ± 0.13
Lignin	24.55 ± 0.19
Ash	5.54 ± 0.22
Extractives	18.24

Note: Three replicates were measured, averaged and evaluated. The extractives composition (%) was calculated by subtracting the holocellulose (%), lignin (%) and ash (%) from 100%

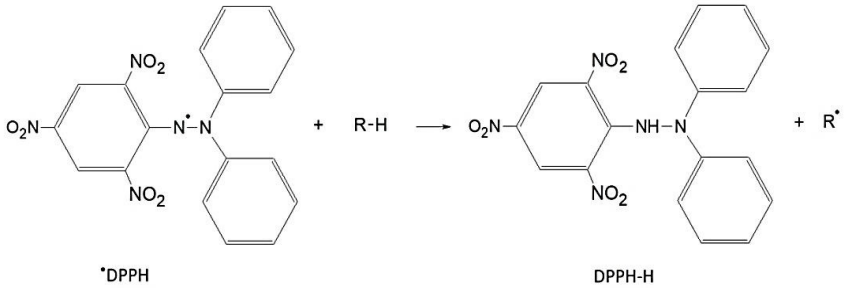


Figure 1: Reduction of •DPPH

The dried and weighed ground bark was added to the DESs at a 1:20 (wt/wt) ratio.

The extraction was performed for 1 h at 60 °C under continuous stirring in a closed flask.

Yield of extractives

The yield of extractives (Y, %) was determined after each experiment by drying the solid residue of bark according to TAPPI T264 cm-97.[33] The yields were determined based on the measured dry matter before and after the extraction, as shown in Eq. 1:

$$Y\ (\%) = 100 \times (m_i - m_{extr}) / m_i \quad (1)$$

where m_i is the mass (g) of the bark before extraction and m_{extr} is the mass (g) of the bark after extraction and drying.

Total phenolic content

The TPC in the extracts was estimated spectrometrically according to Singleton *et al.*[37] with Folin-Ciocalteau's reagent (Fisher Scientific Chemicals, Illkirch, Slovakia) based on the redox reactions of phenols. First, 0.25 g of 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 (λ) 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. The curve was obtained from the absorbance/gallic acid content (g/L) plot. The values of the slope and the intercept of the regression line were 30.574 (standard error = 0.237) and -0.00857 (standard error = 0.00473), respectively. The regression coefficient (R^2) was 0.9997.

Measurement of antioxidant capacity

The antioxidant activity was determined in the form of free radical scavenging activity (RSA) using a method based on the discolouring of the samples after reacting with the stable free 2,2-diphenyl-1-picrylhydrazyl radical (•DPPH) (Fig. 1), and it was measured at a λ of 517 nm.[38]

Briefly, 3.5 to 5.0 mg/mL of the 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 a λ of 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 by Eq. 2:

$$RSA\ (\%) = 100 \times (A_0 - A_{TEST}) / (A_0 - A_{REF}) \quad (2)$$

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.

RESULTS AND DISCUSSION

Extraction yield

Nine DESs were prepared and tested as green solvents for the extraction of spruce bark. The extraction yields obtained using the nine DESs are summarized in Table 2. The yields varied from 11.40% to 27.70%. The greatest yield was obtained for the extraction with DES choline chloride and tartaric acid (27.70%). The lowest yield was achieved using the choline chloride and glycerol eutectic mixture (11.40%). The yield of extractives decreased in the following order of hydrogen bond donors: tartaric acid (27.70%), oxalic acid × $2H_2O$ (27.08%), lactic acid (22.15%), malonic acid (17.94%), citric acid × H_2O (15.17%), malic acid (14.68%), glycolic acid (14.29%), maleic acid (11.87%) and glycerol (11.40%). Based on these results, it was obvious that the yield correlated neither to the number of carboxylic groups nor to the phenolic group of the hydrogen donors, and thus, the number of hydrogen bonds cannot be utilized for the purposeful modification or optimization of the extraction yield.

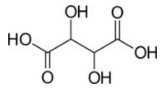
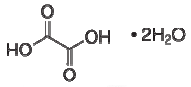
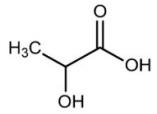
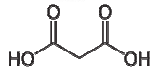
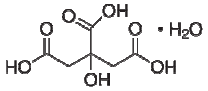
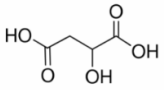
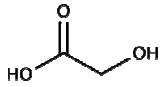
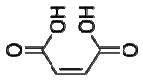
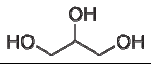
The effect of DESs on extraction and fractionation is not fully understood. The application of DESs to individual components (*e.g.*,

cellulose, lignin) may lead to their solubilisation.[39] When applied to lignocellulosic biomass, its complexity and composition can complicate extraction, fractionation, or delignification. Researches focused on the verification of selectivity in the fractionation process reflect the complexity of DES selectivity.[5-7,40,41] Intermolecular bonds between celluloses, lignins, hemicelluloses and extractives widely complicate extraction processes. Simpler compounds with less complicated structures can be extracted easier and in a shorter time.[6,7,41-43] The delignification of wheat and rice straw takes more than 10 h, but the extraction of chemical substances only takes minutes.[16-18,23]

Extractives from spruce bark contain compounds with high antioxidant activity.[44,45] The antioxidant activity of the extracts and of pure DESs is shown in Figure 2. Extractives from spruce bark showed increased antioxidant activity, compared with the corresponding pure DES. This increase was more pronounced for the extracts from the glycerol, malonic acid, glycolic acid, oxalic acid × $2H_2O$, and tartaric acid DESs than for the malic acid, citric acid × H_2O, lactic acid, and maleic acid DESs.

In the glycerol, glycolic acid, malonic acid, oxalic acid × $2H_2O$, and tartaric acid extracts, the RSA was much higher than for pure DES. For the malic acid, citric acid × H_2O, lactic acid and maleic acid DES extracts, the RSA values were noticeably increased and very similar. The small differences (<5%) were attributed to measurement error. The differences in RSA suggested that each DES preferentially dissolved another type of extractive with a differing reactivity to $^{\bullet}$DPPH. Moreover, each DES had a different extraction yield, and thus, the amount of extractives had an impact on the antioxidant activity and on the reaction with the radical. Table 3 summarizes the antioxidant activity measured at 30 min after the addition of $^{\bullet}$DPPH. The oxalic acid × $2H_2O$ and lactic acid DES extracts had the highest antioxidant activity.

Table 2
Extraction yieldsobtained using different DES systems

DES reagent	Molar ratio	Yield (%)	HBD structure
ChCl:tartaric acid	1:1	27.70 ± 1.69	
ChCl:oxalic acid × $2H_2O$	1:1	27.08 ± 1.88	
ChCl:lactic acid	1:1	22.15 ± 0.68	
ChCl:malonic acid	1:1	17.94 ± 0.63	
ChCl:citric acid × H_2O	1:1	15.17 ± 2.65	
ChCl:malic acid	1:1	14.68 ± 0.86	
ChCl:glycolic acid	1:3	14.29 ± 1.01	
ChCl:maleic acid	1:1	11.87 ± 0.60	
ChCl:glycerol	1:2	11.40 ± 0.04	

Note: ChCl, choline chloride; HBD, hydrogen bond donor

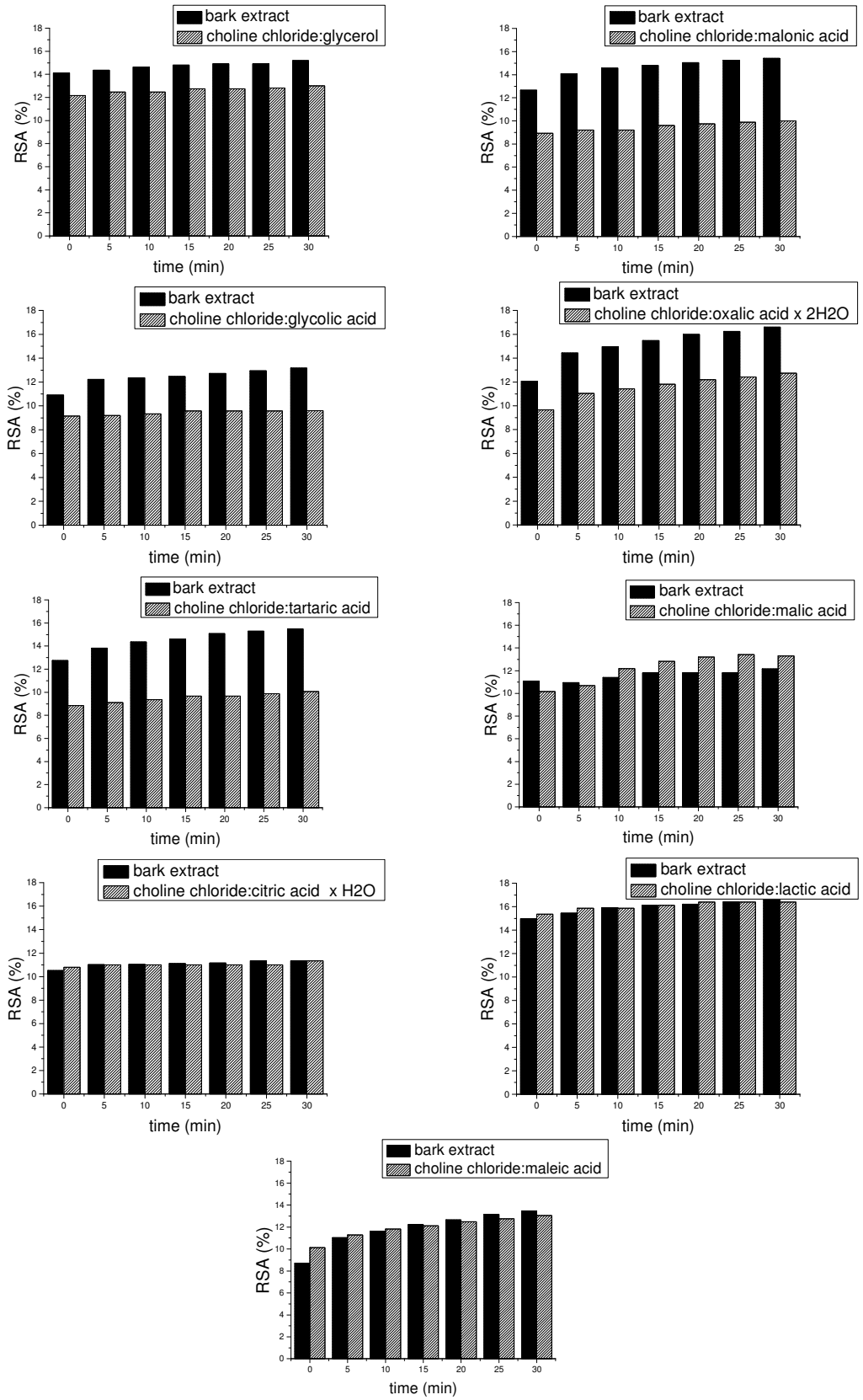


Figure 2: Antioxidant activity of extracts and corresponding pure DESs

Table 3
•DPPH assay of antioxidant activity (RSA) for different extracts after 30 min

Extract	$RSA_{30\ min}$ (%)
ChCl:citric acid × H_2O	11.34
ChCl:malic acid	12.16
ChCl:glycolic acid	13.18
ChCl:maleic acid	13.46
ChCl:glycerol	15.20
ChCl:malonic acid	15.42
ChCl:tartaric acid	15.49
ChCl:lactic acid	16.59
ChCl:oxalic acid × $2H_2O$	16.60

Note: ChCl, choline chloride; standard deviations were ≤ 2.27%

Spruce bark (*Picea abies*) contains a wide variety of compounds with antioxidant properties. Typical antioxidants are phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, lignans and lignins.[46] Conventional extraction methods are based on solvent extraction using ethanol, methanol, chloroform, hexane, or dichloromethane and are generally performed at increased temperature or pressure. Pressurized hot water (subcritical water) can be used for extraction. For non-polar compounds, supercritical carbon dioxide can be used.[47] In previous reports, the antioxidant activities of foodstuffs have been discussed.[38,48,49] Most often, the antioxidant activity of wine,[50,51] or other plants, such as lavender,[52] erythrina,[53] red berries,[54] bitter melon,[55] or buckwheat,[56] is discussed.

The TPC was determined by the Folin-Ciocalteu method and varied between 41 to 463 mg GAE/100 g extract (Fig. 3). Figure 4 shows that the TPC varied between 9 to 100 mg GAE/1 g dry weight (dw), which agreed with previous findings.[57] Agri-food wastes, such as lemon peels, olive leaves, onion wastes and red grape pomace have been investigated as well. The TPC was between 1.53 to 88.03 mg GAE/1 g dw for those wastes. Other studies on European softwood bark extracts reported values in the same range as determined in this work.[58-60]

Following the example of Vasco *et al.*[61], the amount of phenolics in the extracts was classified into three categories: low (<100 mg GAE/100 g extract), medium (100 to 500 mg GAE/100 g extract) and high phenolic content (>500 mg GAE/100 g extract). The extracts classified as low in phenolics were obtained using malic acid, maleic acid and glycerol DESs. Spruce extracts classified as having medium phenolic content were achieved with citric acid × H_2O, oxalic acid × $2H_2O$, tartaric acid, malonic acid, glycolic acid and lactic acid DESs. None of the extracts in this study had high phenolic content.

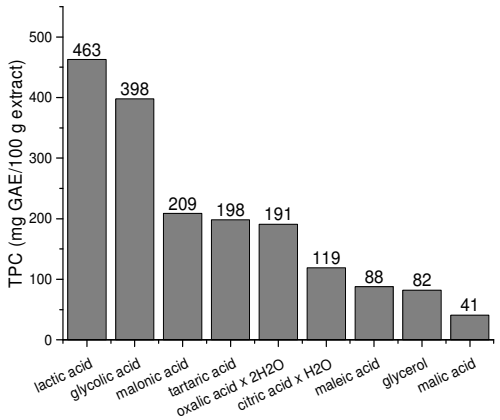


Figure 3: TPC in extracts (mg GAE/100 g extract; standard deviation ≤1%) from choline chloride with each hydrogen bond donor (x-axis)

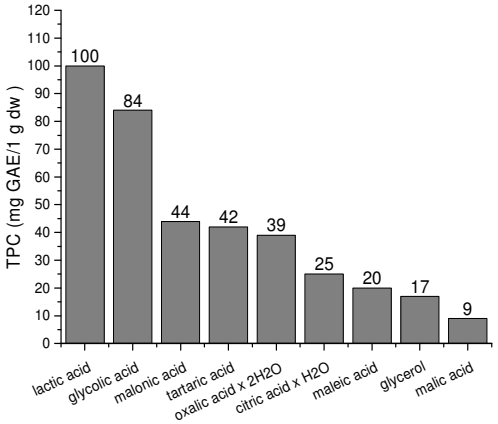


Figure 4: TPC for extracts (mg GAE/1 g dw; standard deviation ≤1%) from choline chloride with each hydrogen bond donor (x-axis);(dw, dry weight)

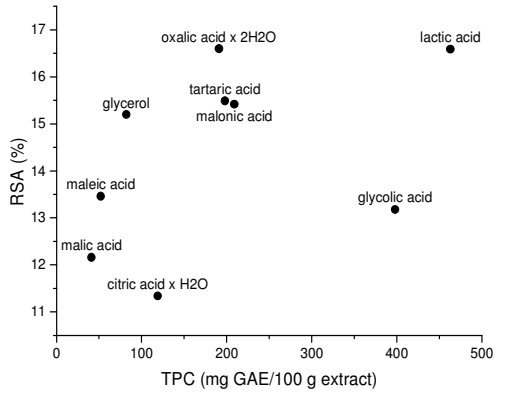


Figure 5: Interaction between antioxidant activity (RSA in 30 min) and TPC of the extracts from choline chloride with hydrogen bond donor DESs

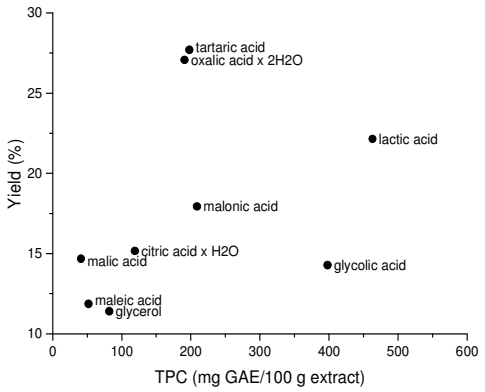


Figure 6: Interaction between extraction yield (%) and TPC of the extracts from choline chloride with hydrogen bond donor DESs

There are conflicting reports on whether there is a correlation between phenolic content and antioxidant activity.[62-66] Figure 5 shows the relationship between the TPC and antioxidant activity. As may be noted in Figure 5, there was no sound correlation between the TPC and antioxidant activity. The correlation coefficient (R^2) was 0.25. There was also no correlation between the yield of extraction and phenolic content (Fig. 6). The highest amount of phenolics (463 mg GAE/100 g extract) was found in the choline chloride and lactic acid DES. The extract from this DES had the highest value of RSA (16.59%), which may have been caused by the different types of phenolic compounds extracted by the different DESs.[67] Thus, the antioxidant activity of the extractants cannot be characterized solely by the TPC and their structural characteristics.[68] Both of the interactions represented in Figures 5 and 6 show that purposefully modifying and optimizing the composition and properties of extractants requires more research. One of the main principles of green chemistry is to develop alternative reaction media, which are the basis of many cleaner technologies. DESs have emerged as promising green solvents to replace conventional solvents,[69] and their ability is demonstrated in the present work.

CONCLUSION

This study was focused on DES systems and their application in spruce bark extraction. DESs were chosen as an environmentally suitable substitution of organic solvents due to their non-flammability and biodegradability.

Nine solvents based on combinations of choline chloride with organic acids and glycerol were used for extraction. The obtained yields from the extraction process showed that there are certain advantages of using DESs as extraction solvents. Due to the complicated composition of the obtained extracts, it was very hard to determine the correlations and connections between the obtained extractives and DESs.

The TPC and antioxidant activity of the extracts were determined. A lower antioxidant activity was observed for the extracts from the citric acid × H_2O (11.34%), malic acid (12.16%), glycolic acid (13.18%) and maleic acid (13.46%) DESs. A remarkably higher RSA (%) was obtained for the extracts from the oxalic acid × $2H_2O$ (16.60%) and lactic acid (16.59%) DESs. The results from the TPC analysis indicated that the extract from the lactic acid DES had the highest content of phenolics (463 mg GAE/100 g extract). Promising results were observed using glycolic acid (398 mg GAE/100 g extract). Moreover, the extraction of spruce bark using DESs was shown to be an efficient method to isolate valuable compounds with high antioxidant activity.

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