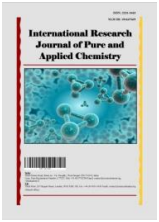

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Influence of Deep Eutectic Solvents (DESs) on Antioxidant and Antimicrobial Activity of Seed Extracts of Selected Citrus Species

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Authors' contributions

This work was carried out in collaboration among all authors. Authors ZA, EH and LK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors EH, LK, DH and BSK performed the analyses of the study. Authors LK and BSK performed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Deep eutectic solvents (DESs), as an alternative to ionic liquids, have greener credentials than ionic liquids and have attracted increasing attention in many applications. Choline chloride-based DESs in combination with different hydrogen bond donors (organic acid, sugars and urea), showed a high extraction efficiency. The aim of this study was to examine the possible effects of choline chloride-based eutectic solvents on the antioxidant and antimicrobial activity of lemon and mandarin orange seed extracts. Lactic acid, glucose, urea and water were used to prepare choline chloride-based Natural Deep Eutectic Solvents (NADESs). Ferric-Reducing Antioxidant Power (FRAP) Assay and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method were used to determine

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antioxidant activity. Antibacterial activity was investigated by diffusion method on reference bacterial strains *E. coli*, *E. faecalis*, *S. aureus*, *B. subtilis* and *L. monocytogenes*. The analysis revealed a significant reduction potential of the eutectic solvent based on lactic acid as well as significant antioxidant activity of lemon and mandarin seed extracts. In general, mandarin extracts showed better antioxidant capacity. *In vitro* antibacterial activity assays showed a complete absence of bacterial growth inhibition of the extracts. However, eutectic solvents with lactic acid have shown a significant antimicrobial effect.

Keywords: Seeds; lemon; mandarin orange; bioactivity; in vitro study.

1. INTRODUCTION

Since ancient times, it has been shown that some Citrus species, belonging to the Rutaceae family, can be used to prevent and help with the treatment of some human diseases [1,2,3,4]. With the development of analytical methods, a detailed examination of Citrus species has been made possible. It has been concluded that citrus fruits, seeds and peel tissues contain many biologically active secondary metabolites such as flavonoids, limonoids, coumarins and furanocoumarins, sterols, volatile oils, organic acids and alkaloids [5,6,7,8,9,10]. Citrus seeds are a good source of limonoids and flavonoids, so they possess greater antioxidant activity than peels [11,12,13,14,15]. Because of its unique aroma and bioactive components content, lemon seed oil might be preferable for functional food, pharmaceutical and cosmetic applications [9,16]. Health-related properties of Citrus species include antiviral, anti-inflammatory, antimicrobial, hepatoprotective effects and many others [15,17,18,19,20]. Some studies have shown that the antioxidant and anti-inflammatory properties of Citrus flavonoids can be helpful against degenerative diseases, particularly brain diseases, as well as cardiovascular diseases and some types of cancer [3,6,18]. Conventional organic solvents and methods are widely used to perform the extraction process of biologically active compounds. However, due to their inherent limitations and the fact that citrus waste also shows an environmental impact, it is important to develop more eco-friendly, efficient, and cost-effective extraction techniques, based on a green extraction approach [5,21]. As a result, diverse extraction techniques have emerged as environmental-friendly alternatives to conventional extraction procedures [22,23,24]. Ionic liquids (ILs) were the first represented green solvents and their newly formed subclass called “Deep Eutectic Solvents” (DESs). A DES is a fluid consisting of hydrogen bond acceptors and hydrogen bond donors. These interactions result in an eutectic mixture with a melting point lower than that of each individual component [25,26,27]. DESs, as an alternative to ionic liquids, have greener credentials than ionic liquids and have attracted increasing attention in many applications such as chemical catalysis, organic synthesis and also extraction and separation processes of various compounds from natural products [28,29,30]. They can be used in combination with microwave assisted extraction, which seems to be a promising tool for the extraction of bioactive compounds, especially in the extraction of phenolic compounds from plants, with the results similar to those obtained by the use of conventional solvents [23,31]. If the eutectic mixture consists of metabolites naturally presented in cells and organisms, newly formed systems are called “Natural Deep Eutectic Solvents” (NADESs). Some of them, such as choline chloride-based NADESs in combination with different hydrogen bond donors (organic acid, sugars and urea), showed a high extraction efficiency compared to conventional methanolic extraction [24,32,33]. Although the use of ionic liquids and DESs/NADESs in separation processes is rapidly increasing, their ecological footprint still has limitations due to possible toxicity and high cost. Other concern is the solid state of most deep eutectic solvents at room temperature [30,31,34]. The aim of this study was to examine the possible effects of choline chloride-based eutectic solvents on the antioxidant and antimicrobial activity of lemon (*Citrus limon*) and mandarin orange (*Citrus reticulata*) seed extracts.

2. MATERIALS AND METHODS

2.1 Plant Material and Chemicals

Lemon and mandarin seeds were collected in the period from December 2019 to June 2020 from commercially available sources. The seeds were washed with water after separation from the fleshy part and dried at room temperature for several days. Before extraction, outer shell of the seeds was removed. The inner part of the seed was then ground in an electric mill and dried at room temperature.

Ultrapure water, prepared with a TKA Smart2Pure device, was used for the extraction process. Choline chloride, methanol, glacial acetic acid, hydrochloric acid, sodium carbonate were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), 2,2`-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and microbiological agents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Iron(II) sulphate heptahydrate and iron(III) chloride hexahydrate were purchased from Honeywell (Charlotte, North Carolina, USA). Folin & Ciocalteu's reagent was purchased from Semikem (Bosnia and Herzegovina). Lactic acid, urea and glucose were obtained from Kemika (Croatia). Reference bacterial strains from the ATCC collection were used for *in vitro* antimicrobial activity testing.

2.2 Preparation of Eutectic Solvents

Lactic acid, glucose, urea and water were used to prepare choline chloride-based eutectic solvents. The initial synthesis of NADES based on glucose was not possible without the addition of water since it is known that glucose-based NADESs with 6 carbon atoms have the highest densities, which is due to a very high density of pure D-glucose. A high density of NADES usually has a negative impact on handling or mixing in chemical processes. The reagents were mixed in different molar ratios shown in Table 1. Choline chloride was dried in an oven at 110°C before use. After mixing the solid reagents, mixtures were placed in a water bath at 80°C (with occasional stirring) until the components were completely dissolved. All prepared eutectic solvents were stable and transparent, with high viscosity.

2.3 Preparation of Extracts

Due to the high density, eutectic solvents with urea and lactic acid were mixed with water in a volume ratio of 2:1 (eutectic solvent:water), except in the case of a eutectic solvent with glucose, where the volume ratio of the mixture was 1:1. Thereafter, 0.2 grams of chopped lemon or mandarin seeds were mixed in a mixture of eutectic solvent: water. The sample was extracted for 1 hour in an ultrasonic bath (Elmasonic S). In order to compare the efficiency of different solvents, extraction with water as control was performed. After extraction, the mixture was centrifuged at 12000 rpm for 2 minutes to precipitate sample particles. The supernatant was then decanted and used for further analyzes.

2.4 Determination of Total Phenolic Content (TPC)

Total phenolic compounds presented in the extracts were quantified spectrophotometrically using the Folin-Ciocalteu test following the protocol [35], with some modifications. 200 µL of extracts was mixed with 2.54 mL of 10% Folin-Ciocalteu reagent. After 5 min 420 µL of 10% sodium carbonate was added. The absorbance of the resulting blue-coloured solution was measured at 765 nm after incubation at room temperature for 1 hour.

2.5 Ferric-Reducing Antioxidant Power (FRAP) Assay

The reducing powers of the extracts that reflected their antioxidant activity were determined following the protocol [36]. 3 mL of prepared FRAP reagent was mixed with 100 µL of extracts. Absorbance at 593 nm was recorded after 30 min incubation at 37 °C. The FRAP value was calculated from the calibration curve of iron(II) sulfate heptahydrate ($y = 0{,}001x + 0{,}0698$; $R^2 = 0{,}9997$).

2.6 DPPH Radical Scavenging Activity

2,2-diphenyl-1-picryl-hydrazyl (DPPH) method was performed according to earlier described method [37]. 100 µL of extract was mixed with 1900 µL of methanol and 500 µL of 0.5 mM DPPH radical solution. The absorbance was measured at 517 nm with methanol as a blank sample. 0.5 mL of 0.5 mM DPPH dilution, diluted with 4 mL of methanol, was used as a control sample. The radical scavenging effect (%) or percent inhibition of DPPH radical was calculated according to the equation: $[(Ac - As) / Ac] \times 100$ where As is the absorbance of the solution containing the sample at 517 nm and Ac is the absorbance of the DPPH solution.

2.7 *In vitro* Antimicrobial Activity Testing

Antibacterial activity was investigated by diffusion method on reference bacterial strains *E. coli* (EC), *E. faecalis* (EF), *S. aureus* (SA), *B. subtilis* (BS) and *L. monocytogenes* (LM). From the microorganisms strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared. The strains were then placed on the surface of the nutrient substrate Mueller-

Hinton agar, dispersed in sterile Petri dishes. Substrate thickness was 4 mm. In the agar sterile drill-shaped holes were made (diameter 6 mm), into which 50 µL of eutectic solvent or extract were added. Plates were incubated at 37°C for 24 h. After the incubation period, the size of the inhibitory zone was measured.

3. RESULTS AND DISCUSSION

3.1 Total Phenol Content (TPC)

Total phenolic content could not be determined and quantified spectrophotometrically through the Folin-Ciocalteu test. The reason for this was the occurrence of precipitation reactions after the addition of Folin & Ciocalteu's reagent to the citrus seed extracts prepared using NADESs. Fig. 1 shows the precipitation reactions with eutectic solvent (left) and citrus extract in eutectic solvent (right). In the case of aqueous extracts, the appearance of precipitates by the addition of Folin & Ciocalteu's reagent was not recorded, so it can be concluded that the reason for the occurrence of precipitating reactions is eutectic solvents.

3.2 Antioxidant Activity *In vitro*

Antioxidant capacity of lemon and mandarin seed extracts measured using DPPH and FRAP methods are shown in Table 2. and Table 3. According to FRAP results, extracts obtained with Choline chloride:Glucose:Water eutectic solvent (ChCl:Glu:Water) have the highest efficiency and ability to reduce ferric ions. Slightly higher values were recorded for mandarin seed extracts with a value of 2356.5 µmol/L, compared to lemon seed extracts with the value of 1553.0 µmol/L. High values of aqueous extracts have been measured, which justifies addition of water to the NADESs in order to improve the extraction efficiency [24]. The presence of water decreases the viscosity and affects polarity [38] and these properties are important for extraction efficiency. High extraction of bioactive components with NADES can be related to the hydrogen bonds formed between these two components [39].

Obtained FRAP and DPPH results for citrus seed extracts, prepared with NADES containing choline chloride and urea with molar ratio 1:1 showed that urea is an adequate solvent for the preparation of eutectic solvents with choline chloride. Urea is one of the amides with the highest affinity for the formation of hydrogen bonds [26]. By increasing the concentration of urea, hydrogen interactions between choline cations and Cl^- anions decreased, while those among urea molecules increased. It was found that choline chloride and urea ratio of 1:2 is necessary for a reasonable strength of hydrogen bond interaction to maintain the low melting point of the mixture of choline chloride with urea [40]. In a mixture of choline chloride and urea using molar ratio of 1:2, good solubility of salts, aromatic acids and amino acids was observed [26]. Interesting aspect is that highest solubility is not always correlating with highest extractability of a compound [41]. This is seen by lower FRAP and DPPH values obtained using ChCl:U1 with the molar ratio 1:2, compared to ChCl:U with the molar ratio 1:1.

Ultrasonic extraction with lactic acid:choline chloride mixture with molar ratio of 3:1, prepared as 80% (v/v) aqueous solutions, showed an exceptionally efficient method for polyphenol recovery from selected medicinal plants (dittany, fennel, marjoram, mint and sage) [42]. ChCl:LA extract with molar ratio 1:1 showed significantly lower efficiency compared to those from the mentioned study, which makes it the least effective in this test. Based on this, it can be concluded that a 1:1 ratio is not adequate for this NADES. By changing the molar ratio of ChCl:LA1 to 1:2, the FRAP and DPPH efficiency can be increased.

Table 1. Composition of natural deep eutectic solvents (NADESs)

NADES Composition	Abbreviation	Molar Ratio
Choline chloride : Urea	ChCl : U	1 : 1
Choline chloride : Urea	ChCl : U1	1 : 2
Choline chloride : Lactic acid	ChCl : LA	1 : 1
Choline chloride : Lactic Acid	ChCl : LA1	1 : 2
Choline chloride : Glucose : Water	ChCl : Glu : W	2 : 1 : 1

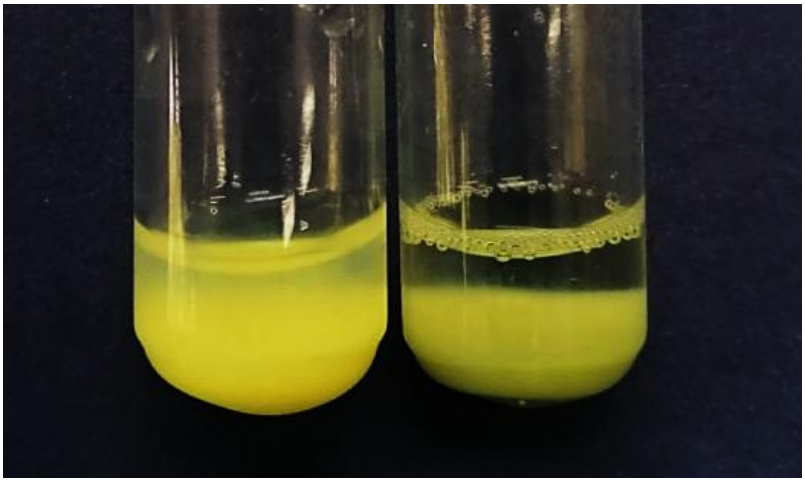

Fig. 1. Precipitation reactions recorded by addition of Folin & Ciocalteu's reagent

Table 2. FRAP and DPPH method results for lemon seed extracts

NADES composition	FRAP [µmol/L]	DPPH [% inhibition]
ChCl:U	1369.5	46.80
ChCl:U1	1144.4	21.00
ChCl:LA	1052.7	13.21
ChCl:LA1	1217.5	22.38
ChCl:Glu:Water	1553.0	69.45
Water	1297.0	25.67

Table 3. FRAP and DPPH method results for mandarin orange seed extracts

NADES composition	FRAP [µmol/L]	DPPH [% inhibition]
ChCl:U	1971.8	57.47
ChCl:U1	1106.7	45.03
ChCl:LA	936.2	41.76
ChCl:LA1	1336.7	49.91
ChCl:Glu:Water	2356.5	92.93
Water	1831.3	54.68

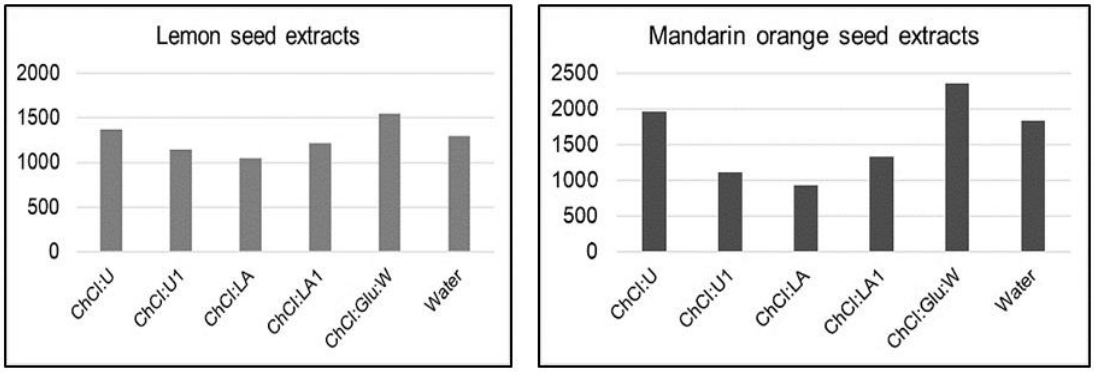

Fig. 2. Graphical representation of reduction ability of extracts obtained by FRAP method

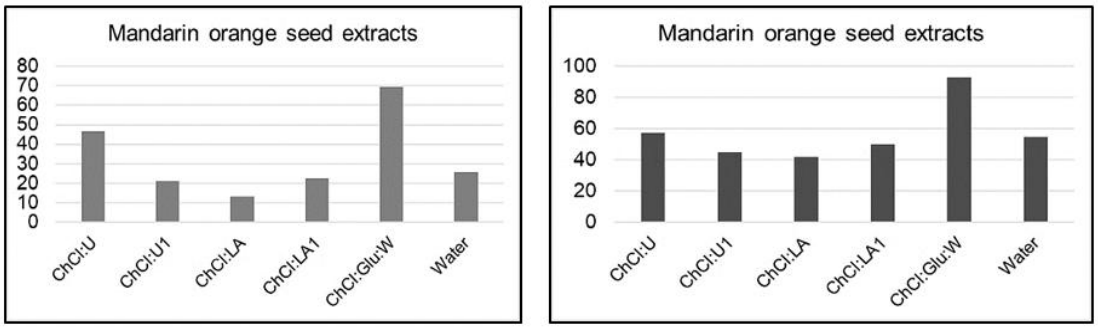

Fig. 3. Graphical representation of DPPH radical inhibition

DPPH results showed the same pattern as FRAP method. The highest antioxidant activity was measured for the extract obtained with ChCl:Glu:Water, with a value of 92.93% for mandarin extract and 69.45% for lemon extract. The lowest values were observed for ChCl:LA extracts. Based on the results of DPPH method, it is noted that all mandarin seed extracts showed significantly higher antioxidant activity compared to lemon seed extracts.

3.3 Antimicrobial Activity *In vitro*

The results obtained by the diffusion method used for *in vitro* testing of antimicrobial activity of eutectic solvents and citrus seed extracts are shown in Table 4 and Table 5.

The analysis showed antimicrobial activity strains *E. coli*, *E. faecalis*, *S. aureus*, *B. subtilis* and *L. monocytogenes* of both NADESs that contain choline chloride and lactic acid. Slightly higher zones of inhibition were observed for ChCl:LA1, with the molar ratio 1:2, compared to ChCl:LA with the molar ratio 1:1. It is significant to note that only organic acid-based NADESs show antimicrobial activity. This may be related to their high polarity and strongly acidic properties, by which they differ from sugar- and urea-based NADESs. Sugar-based NADESs are weakly acidic in water, while NADESs containing urea possess basic properties [43]. Studies have shown that microorganism's growth can be inhibited due to high acidity of NADESs, which damages the cell membrane and its proteins. In addition, the absence of antimicrobial activity in NADES containing glucose may be related to the fact that some microorganisms use glucose as a nutrition source.

For all extracts, a complete absence of antibacterial activity was recorded. Compared to the eutectic solvent itself, the extracts do not show antibacterial activity against *E. coli*, *E. faecalis* and *S. aureus*, but also reduce the inhibitory ability of the eutectic solvent itself.

Table 4. Antibacterial activity of NADESs

NADES composition	Inhibition zone [mm]				
	EC	LM	BS	EF	SA
ChCl:U	-	-	-	-	-
ChCl:U1	-	-	-	-	-
ChCl:LA	23	36	26	35	25
ChCl:LA1	30	45	28	40	27
ChCl:Glu	-	-	-	-	-

Table 5. Antibacterial activity of lemon and mandarin orange seed extracts

Extract	NADES composition	Bacterial strain				
		EC	LM	BS	EF	SA
Lemon	ChCl:LA	19	35	24	32	20
	ChCl:LA1	24	40	26	35	21
Mandarin orange	ChCl:LA	20	33	22	31	24
	ChCl:LA1	24	39	24	32	25

4. CONCLUSION

DESs can be used as safe, efficient, simple, and low–cost solvents. In this work, choline chloride-based eutectic mixtures were prepared and used for extraction of bioactive components from citrus and mandarin seeds. Antioxidant and antimicrobial activity of seed extracts obtained with different DESs were compared. Extracts obtained using Choline chloride:Glucose:Water eutectic solvent (ChCl:Glu:Water) showed highest antioxidant activities. It can be concluded that addition of water to the NADESs improved the extraction efficiency. None of the extracts showed antibacterial activity against tested strains. Antibacterial activity of the extracts obtained using choline chloride and lactic acid is attributed to eutectic solvent. Further work is needed to identify bioactive components of seed extracts and analyze influence of DESs extraction solvents on bioactive components content.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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