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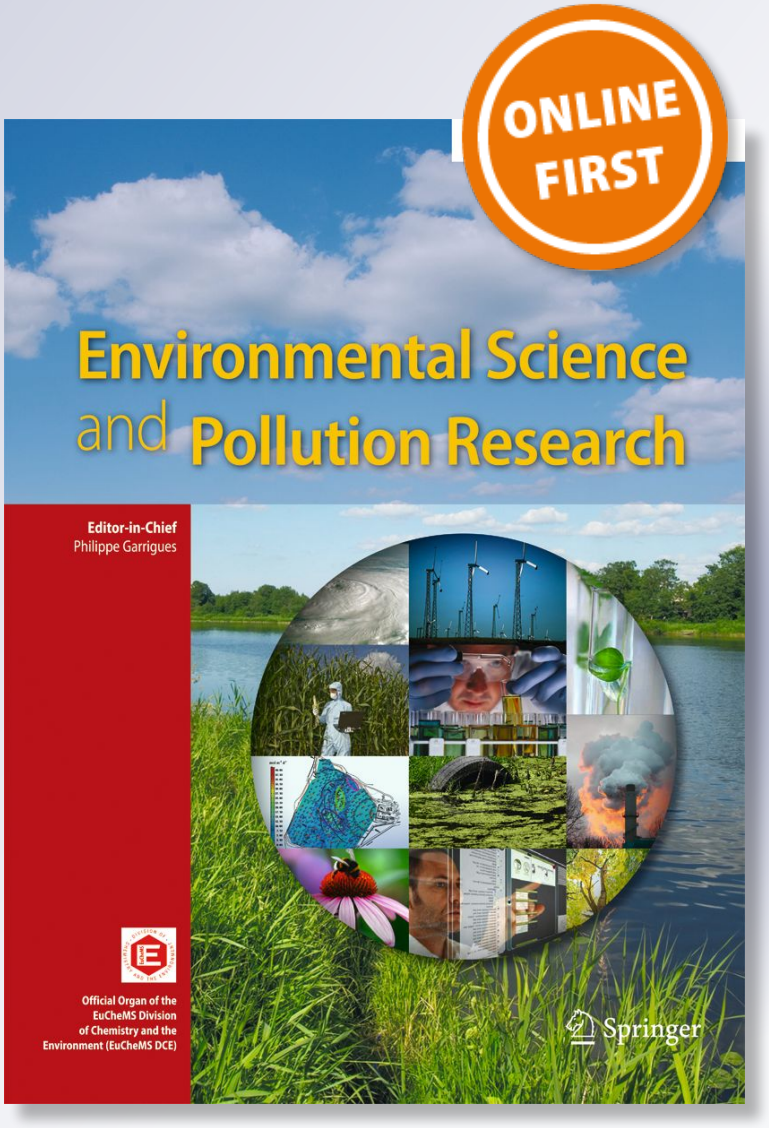
Environmental Science and Pollution Research

ISSN 0944-1344

Environ Sci Pollut Res
DOI 10.1007/s11356-018-1669-z



Springer

Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-018-1669-z

RESEARCH ARTICLE

Antimicrobial, cytotoxic and antioxidative evaluation of natural deep eutectic solvents

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Received: 21 November 2017 / Accepted: 1 March 2018

Abstract

Natural deep eutectic solvents (NADES) are a new generation of green solvents. They are mixtures of two or three compounds such as choline chloride as a cationic salt and alcohols, acids, amides, amines or sugars as hydrogen-bond donors. Although the majority of NADES' components are of natural origin and therefore NADES are often presumed to be non-toxic, the evaluation of their toxicity and biodegradability must accompany the research on their synthesis and application. Therefore, the aim of this work was to investigate the effect of ten synthesised NADES towards bacteria (i.e., *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), yeast (i.e., *Candida albicans*) and human cell lines (i.e., HeLa, MCF-7 and HEK293T). In addition, oxygen radical absorbance capacity (ORAC) method was used to determine the antioxidative activity of the tested NADES. Differences in toxicity response between microorganisms and cell lines were observed, and only NADES that contained organic acid showed toxicity towards the test systems. Furthermore, the NADES containing compounds that possess antioxidative activity also showed antioxidative activity. However, research whose primary purpose is the synthesis and application of NADES must be followed by an evaluation of their biological properties (e.g., antimicrobial activity, toxicity towards animal cells and antioxidative or other biological activity) to find the solvent with the best profile for wider industrial applications.

Keywords Antimicrobial activity · Antioxidative activity · Cytotoxicity · Natural deep eutectic solvents · ORAC

Introduction

The development of green technologies is highly oriented towards designing new solvents with safer ecotoxicological profiles, lower costs and desirable properties for different technological processes (Matzke et al. 2010). Since the advent of ionic liquids (ILs) a few decades ago, their potential application and their environmental fate and toxicity have been intensively studied and reported (Abbott et al. 2004; Alirezaei et al. 2015). Despite their promising potential for various processes, numerous studies have shown that ILs are not intrinsically environmentally friendly as they are toxic or even more toxic in comparison to organic solvents (Egorova and Ananikov 2014). In recent years, a new class of green solvents, deep eutectic solvents (DES), have attracted much attention as alternative to conventional organic solvents and ILs (Hayyan et al. 2015). DES are mixtures of two or more compounds with a melting point lower than that of each component. In comparison to other solvents, they possess many desirable physicochemical properties, including non-flammability, viscosity, biodegradability and the potential to be customised for particular purposes (Paiva et al. 2014). Since their emergence, DES have attracted attention as solvents for organic synthesis and (bio) catalysis, polymer production, electrochemistry, nanomaterials, separation processes and

Highlights
- Differences in natural deep eutectic solvents (NADES) toxicity responses between bacteria and cell lines were observed.
- NADES containing organic acid possess toxicity.
- BMaPro had a beneficial effect on the proliferation of all tested cell lines.
- NADES could possess antioxidative activity.

Responsible editor: Philippe Garrigues

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Published online: 09 March 2018

analysis and biomedical applications as reported by Hayyan et al. (2016). Different authors have shown that DES are eco-friendly, biodegradable and non-toxic (Abbott et al. 2004; Radošević et al. 2015; Juneidi et al. 2016). However, some of DES' toxic effects indicate that their individual components can possess a different toxicity profile from that of the mixture (Hayyan et al. 2013).

Natural deep eutectic solvents (NADES) represent a subclass of DES. They are mixtures of two or three natural compounds, such as choline chloride (as hydrogen-bond acceptors, HBA) and alcohols, acids, amides, amines or sugars (as hydrogen-bond donors, HBD). Although the majority of NADES-forming components are of natural origin and are consequently often presumed to be non-toxic, an evaluation of their toxicity and biodegradability must accompany their synthesis and application. Recently, Paiva et al. (2014), Zhao et al. (2015) and Hayyan et al. (2016) characterised the toxicity profiles of choline chloride-based NADES in cell lines and microorganisms, indicating different degrees of toxicity depending on the NADES' constitutive components. Paiva et al. (2014) studied the cytotoxic effects of 11 different NADES on fibroblast-like L929 cells and showed pronounced toxic effects of NADES with acids (i.e., tartaric and citric acid). Zhao et al. (2015) reported the harmful effects of acid-based DES on Gram-negative and Gram-positive bacteria, while Hayyan et al. (2016) emphasised the important impact of organic acid as a NADES HBD on the solvents' toxicity profiles in human cell lines. The applicability of novel solvents for commercial processes is still in the initial phases of research and development (Abbott et al. 2007). Prior to their industrial application in different technological processes, it is essential that NADES are well characterised, including their biological properties.

For the purpose of this work, ten different NADES with potential applications in the extraction of biological compounds and/or biocatalysis were synthesised and tested for their biological properties (Cvjetko Bubalo et al. 2015, 2016; Radošević et al. 2016). The prepared NADES are illustrated in Table 1, containing choline chloride, betaine and citric acid as HBAs with various HBDs, including organic acid, sugar, sugar alcohol, amino acid and amide. A compound's inhibition of bacterial growth is a common toxicological assessment method for characterising its toxicity profile in a short period of time and with low costs. The antimicrobial activity of tested NADES was studied in Gram-positive bacteria (i.e., *Staphylococcus aureus*), Gram-negative bacteria (i.e., *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) and yeast (i.e., *Candida albicans*). These microorganisms were selected as the most common cause of health problems, and they are usually used for the toxicity testing of different substances (Jardim et al. 1990; Chen et al. 2010; Fadli et al. 2011; Spaulding et al. 2012; Young et al. 2012; Fu et al. 2013).

Table 1 List of the tested NADES

NADES	Abbreviation	Molar ratio	Water content (%, *w*/*w*)
Choline chloride:oxalic acid	ChOx	1:1	10
			30
			50
Choline chloride:urea	ChU	1:2	10
			30
			50
Choline chloride:xylitol	ChXylol	5:2	10
			30
			50
Choline chloride:sorbitol	ChSol	2:3	10
			30
			50
Betaine:glucose	BGlc	5:2	10
			30
			50
Betaine:malic acid:proline	BMaPro	1:1:1	10
			30
			50
Betaine:malic acid:glucose	BMaGlc	1:1:1	10
			30
			50
Citric acid:proline	CitPro	1:1	10
			30
			50
Citric acid:glucose:glycerol	CitGlcGly	1:1:1	10
			30
			50
Citric acid:fructose:glycerol	CitFruGly	1:1:1	10
			30
			50

The cytotoxicity profiles of the selected NADES were also investigated in human cell lines (i.e., HEK293T, HeLa and MCF-7) using the commercial CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay. In addition, the antioxidative activity of the selected NADES by the ORAC method was also determined.

Materials and methods

Chemicals

Chemicals for NADES syntheses (choline chloride CAS 67-48-1; betaine CAS 107-43-7; citric acid CAS 5949-29-1; malic acid CAS 6915-15-7; oxalic acid CAS 144-62-7; glucose CAS 50-99-7; fructose CAS 57-48; xylitol CAS 87-99-0;

sorbitol CAS 50-70-4 and glycerol CAS 56-81-5) were purchased from Sigma Aldrich. The purity of used chemicals was ≥ 99%, and they were used without further purification.

Preparation of NADES

In this study, the heating method was used for preparation of NADES in certain molar ratios as listed in Table 1 and described previously by Radošević et al. (2015). Briefly, before use, choline chloride was dried in the vacuum concentrator (Savant SPD131DDA Speed Vac Concentrator, Thermo Scientific, USA) at 60 °C for 24 h. The two or more components in specific ratios with 10, 30 or 50% (*w*/*w*) of water were placed in glass flask with round bottom with stirring bar. The mixture was heated on temperature between 50 and 70 °C for 2–6 h until the clear homogeneous liquid was formed.

Antimicrobial activity test

Bacterial and yeast strains of *E. coli* 3014, *P. mirabilis* 3008, *S. typhimurium* 3064, *P. aeruginosa* 3024, *S. aureus* 3048 and *C. albicans* 86 were obtained from the Collection of Microorganisms of the Laboratory of General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb (Zagreb, Croatia). The bacterial cultures were stored at − 70 °C in nutrition broth (Biolife, Milano, Italy) while *C. albicans* was stored in yeast extract-peptone-glucose (YPG) medium composed of 2% glucose, 1% yeast extract and 1% peptone (pH 5) with 30% (by volume) glycerol, respectively. The strains were activated in the same corresponding broths and maintained at 4 °C.

Antimicrobial activity of NADES was tested to selected microorganisms using disk diffusion method. Microbial cultures were prepared using McFarland turbidity (Densimat, bioMérieux, Marcy I'Etoile, France) with 0.4 standard (10^7–10^8 CFU mL^{-1}). Bacterial and yeast suspensions (0.1 mL) of each microorganism (*S. typhimurium.*, *E. coli*, *P. aeruginosa*, *P. mirabilis*, *S. aureus and C. albicans*) were smeared on the nutrient agar plate for bacteria and malt agar for yeast and left to dry. Sterile 6-mm filter disks (Macherey-Nagel GmbH, Germany) were immersed in appropriate NADES placed on the surface of agar inoculated with test microorganism. Antimicrobial activity of NADES forming component was also tested in concentration range of 0.2–0.001 mg L^{-1}. After incubation on 37 °C during 24 h, inhibition was detected as clear halo diameter (mm).

Cytotoxicity assay

Three adherent human cell lines, obtained from the Ruđer Bošković Institute (Zagreb, Croatia), were used in this work. HeLa cell line derived from the cervical adenocarcinoma (ATCC No. CCL-2™), MCF-7 cell line derived from breast adenocarcinoma (ATCC no. HTB-22) and HEK293T cell line derived from embryonic kidney (ATCC No. CRL-3216™) were cultured in DMEM (Dulbecco's modified Eagle's medium, Lonza, Belgium) supplemented with 5% heat-inactivated FBS (fetal bovine serum, Gibco, UK) and maintained in T-flasks in the incubator with humidified atmosphere and 5% CO_2 at 37 °C.

The effects of synthesised NADES on cell viability were examined by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay (Promega, USA) according to the manufacturer's instructions. Briefly, HeLa, MCF-7 and HEK293T cells were seeded in 96-well plates at a density of 3×10^4 to 5×10^4 cells per well in 100 μL of culture media. After overnight incubation, HeLa, MCF-7 and HEK293T cells were treated with NADES (Table 1) in the nominal concentrations of 500–2000 mg L^{-1}. After 72 h of exposure, 10 μL of the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation reagent was added to each well and cells were incubated for further 3 h. Then, absorbance at 490 nm was read by the microplate reader (Tecan, Switzerland). The experiments were performed in triplicate and data were expressed as the means ± S.D. Cell viability was calculated as percentage of treated versus control cells. Corresponding EC_{50} values were calculated from the dose-response curves from equations of best-fitted trend lines.

Antioxidative activity of NADES determined by ORAC assay

ORAC values were determined according to method described by Ninfali et al. (2005). Fluorescence was measured by a Varian Cary Eclipse Spectrofluorimeter (Palo Alto, CA, USA). The final reaction mixture for the assay (3 mL) was prepared as follows: 2.25 mL of 0.04 mL fluorescein sodium salt in 0.075 M sodium phosphate buffer (pH 7.0), and 0.375 mL diluted NADES or 25 mL Trolox as standard. The reaction mixtures were incubated for 30 min at 37 °C followed by reaction initiation with 0.375 mL 152 mm AAPH. Fluorescence was read every minute up to value zero at 485 nm excitation and 520 nm emission. The negative control was 0.075 M sodium phosphate buffer. Results were calculated as ORAC values using the differences of areas under fluorescein decay curve between the blank and the sample. The results were expressed as μmol Trolox equivalent per gram of NADES (μmol TE g^{-1}).

Results and discussion

Antimicrobial activity of NADES

Table 2 shows the antimicrobial activity of the tested NADES towards the most common pathogen bacteria and yeast strains, which was determined using a disk diffusion assay. All

Table 2 Antimicrobial activity of NADES with different water content to the most common pathogens (mm)

NADES	Water content % (*w*/*w*)	*Escherichia coli*	*Proteus mirabilis*	*Salmonella typhimurium*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
ChOX*	10	47 ± 2	49 ± 1	45 ± 4	50 ± 2	73 ± 3
	30	44 ± 3	–	–	49 ± 1	55 ± 3
	50	43 ± 3	–	–	49 ± 4	50 ± 2
ChU	10	37 ± 5	–	–	–	–
	30	–	–	–	–	–
	50	–	–	–	–	–
ChXyol	10	–	–	–	–	–
	30	–	–	–	–	–
	50	–	–	–	–	–
ChSol	10	–	–	–	–	–
	30	–	–	–	–	–
	50	–	–	–	–	–
BGlc	10	–	–	–	–	–
	30	–	–	–	–	–
	50	–	–	–	–	–
BMaGlc	10	44 ± 2	62 ± 3	24 ± 3	45 ± 3	49 ± 2
	30	22 ± 1	45 ± 4	27 ± 3	38 ± 2	45 ± 3
	50	–	47 ± 2	–	30 ± 5	44 ± 5
BMaPro	10	28 ± 3	73 ± 1	30 ± 2	50 ± 1	51 ± 3
	30	23 ± 4	65 ± 4	28 ± 3	45 ± 1	47 ± 1
	50	–	44 ± 2	34 ± 1	28 ± 3	44 ± 4
CitPro	10	49 ± 1	47 ± 4	33 ± 4	44 ± 5	50 ± 2
	30	44 ± 4	56 ± 2	37 ± 3	45 ± 4	46 ± 3
	50	33 ± 5	44 ± 5	26 ± 5	24 ± 2	44 ± 3
CitGlc-Gly	10	46 ± 2	68 ± 1	44 ± 2	50 ± 1	48 ± 5
	30	34 ± 2	83 ± 3	51 ± 3	47 ± 5	46 ± 2
	50	22 ± 5	49 ± 5	57 ± 5	44 ± 3	44 ± 1
CitFruGly	10	50 ± 4	81 ± 2	55 ± 1	51 ± 4	51 ± 3
	30	45 ± 1	76 ± 2	42 ± 3	43 ± 3	48 ± 3
	50	38 ± 2	50 ± 3	37 ± 5	28 ± 2	43 ± 2

**Candida albicans* was only inhibited by ChOX as follows: 48 ± 3 mm (water content 10%), 23 ± 2 mm (water content 30%), 25 ± 1 mm (water content 50%)

NADES except ChXylol, ChSol and BGlc inhibited the growth of most of the tested microorganisms, indicating that choline, betaine, sugar and sugar alcohols as forming compounds of NADES do not influence the possible antimicrobial activity of NADES. Also, ChU at 10% of water content was effective only against *E. coli* (37 ± 5 mm), indicating the low toxicity of urea towards the tested microorganisms. On the other hand, NADES containing citric acid showed growth inhibition, with the highest growth inhibition detected for CitGlcGly (22 ± 5 to 83 ± 3 mm) and CitFruGly (28 ± 2 to 81 ± 2 mm), indicating their potential use as antimicrobial agents. For instance, BMaPro (23 ± 4 to 73 ± 1 mm) and BMaGlc (22 ± 1 to 62 ± 3 mm) also showed inhibitory effects on pathogens but at lower levels compared to CitGlcGly and CitFruGly. We studied different concentrations of water content in the tested NADES (10, 30 or 50%, *w*/*w*) since pure NADES are not commonly used in exaction and/or biocatalysis processes due to their viscosity. On the other hand, diluted NADES with over 50% water content can break the halide-HBD supramolecular complex, and a simple aqueous solution of the individual components could be obtained (Gutierrez et al. 2009). Previous publications also indicated that NADES containing organic acids possess higher antimicrobial activity than alcohol-, amine- and sugar-based NADES. According to the literature, the introduction of an additional hydroxyl group to organic acids as the HBD of the DES increases its antibacterial activity (Zhao et al. 2015; Wikene et al. 2017). The toxicity of the various DES could also be associated with their pH values. For example, organic acid-based NADES are the most polar, whereas both sugar- and

polyalcohol-based NADES are the least polar (Dai et al., 2013). Furthermore, NADES in water are weakly acidic (sugar- and sugar alcohol-based NADES, pH ≈ 4.5), while carboxylic acid-containing NADES are strongly acidic (pH < 3), with NADES containing oxalic acid as the most acidic, followed by NADES containing citric and malic acid as the second and third most acidic, respectively. Only NADES containing urea possess a basic characteristic (pH ≈ 9; Cvjetko Bubalo et al. 2015; unpublished data). Similar behaviour has been observed in other studies, where microorganism's growth was inhibited due to high acidity, which damages the cell membrane and its protein (Stratford et al. 2009; Gauniya et al. 2010; Juneidi et al. 2016).

All tested microorganisms except *C. albicans* showed relatively high sensitivity to the tested NADES, where the highest inhibition was detected for *P. mirabilis* (83 ± 3 mm). ChOX at 10% of water content inhibited all pathogens including *C. albicans*, while at 30 and 50% of water content was not toxic only for *P. mirabilis* and *S. typhimurium*. ChOX inhibited the Gram-positive bacteria *S. aureus* (73 ± 3 mm) the most. Some reports have noted that NADES and DES increase the permeability of the lipid membrane of eukaryotic cells (Hayyan et al. 2015; Mbous et al. 2017), but to the best of our knowledge, their effect on bacterial membranes is still unknown, although it could depend on their solubility of a broad range of solutes, such as components in the bacterial membrane; pH value; osmolality; or chelation of membrane-bound divalent cations (Wikene et al. 2017). However, we presume that obtained results could be explained by the presence of peptidoglycan, a polysaccharide backbone that consists of *N*-acetylmuramic acid and *N*-acetylglucosamine residues cross-linked by short peptides. NADES may be partially dissociated in aqueous solutions, and the cholinium cation in NADES can interact with polysaccharide chains through hydrogen bonding or electrostatic interaction, which leads to cell wall disruption (Wen et al. 2015). Since Gram-negative bacteria possess an extra outer lipopolysaccharide membrane on the cell wall, they are less permeable. This could be the reason why ChOX inhibited *E. coli* (47 ± 2 mm), *P. mirabilis* (49 ± 1 mm), *S. typhimurium* (45 ± 4 mm) and *P. aeruginosa* (50 ± 2 mm) less in comparison to *S. aureus* (73 ± 3 mm).

The antimicrobial effects of NADES' single components were also tested, but only oxalic, malic and citric acid showed antimicrobial activity, indicating once more that NADES with organic acids could have antimicrobial activity (Fig. 1).

This is not unexpected since other tested compounds are known for their low toxicity towards microorganisms, and some of them, such as glucose, fructose and glycerol, are used as nutrition sources by several bacteria and fungi (Silva et al. 2010; Juneidi et al. 2016). Also, all acids showed lower growth inhibition in comparison to synthesised NADES. The results can be explained by synergistic effect of forming NADES as previously reported by Hayyan et al. (2013). Oxalic acid showed the highest inhibition of pathogen growth, followed by malic acid and citric acid. NADES-forming compounds possess different degrees of antimicrobial activity than the NADES themselves. As observed, citric acid was the least toxic, and citric-based NADES had the highest toxicity. According to the literature, DES are more toxic in comparison to their individual components due to delocalised charges as a result of the hydrogen bond, especially in acid-based DES (Hayyan et al. 2013; Zhao et al. 2015).

Cytotoxicity of NADES in cell cultures

Most of the NADES toxicity profiles reported in the current literature were determined using bacteria such as *E. coli*, *S. aureus*, *Salmonella enteritidis*, *Listeria monocytogenes*, and *Vibrio fischeri*, some fungi and different cell lines. The majority of the studied NADES were based on choline chloride since choline, as a component of vitamin B, possesses an important role in cellular metabolism (Florindo et al. 2014). In this study, four different NADES based on choline chloride and oxalic acid (OX), urea (U), xylitol (Xyol) and sorbitol (Sor) as HBDs were tested in HeLa, MCF-7 and HEK293T cells with the CellTiter 96® AQ_{ueous} Proliferation Assay in the concentration range of 500–2000 mg L^{-1} (Table 3). The highest inhibitory effect was observed for ChOX in tumour HeLa and MCF-7 cells (EC_{50} of 330.90 ± 29.75 and 558.98 ± 54.32 mg L^{-1}, respectively), while its effect in normal HEK293T cells (EC_{50} > 2.000 mg L^{-1}) was one order of magnitude higher than in tumour cells. This finding is in agreement with our previous results (Radošević et al. 2015), where the formation of calcium oxalate crystals inside the cells induced detrimental effects on both tumour and normal cells. The stronger toxic effect in tumour cells than in normal cells can be explained by the fact that tumour cells, due to higher energy demands, uptake more culture media ingredients (i.e., glucose, amino acids, salts, growth factors) and ChOX as well. Again, the results from the tested cell lines confirmed the influence of organic acid as an HBD on overall NADES toxicity and showed consistency with previously reported results (Paiva et al. 2014; Zhao et al. 2015; Hayyan et al. 2016).

A pronounced cytotoxic effect of ChU was observed only in MCF-7 cells, where an EC_{50} of 83.50 ± 7.94 mg L^{-1} was obtained (Table 3). At the same time, EC_{50} values of > 2000 mg L^{-1} were obtained for HeLa and HEK293T cells. The toxic effect of ChU in MCF-7 cells could be caused by its high pH value (~ 9.00; Cvjetko Bubalo et al. 2015) since the optimal pH for cell cultivation is in the neutral range (7.0–7.4). However, the viability of HeLa and HEK293T cells was not affected by ChU, indicating a cell-type-dependent cytotoxicity. ChXyol and ChSol did not cause cytotoxic effects in both normal and tumour cells since 50% inhibition of cell growth was not obtained, indicating their low toxicity profile. From the metabolic point of view, carbohydrates (mostly glucose and fructose) are utilised as the carbon and

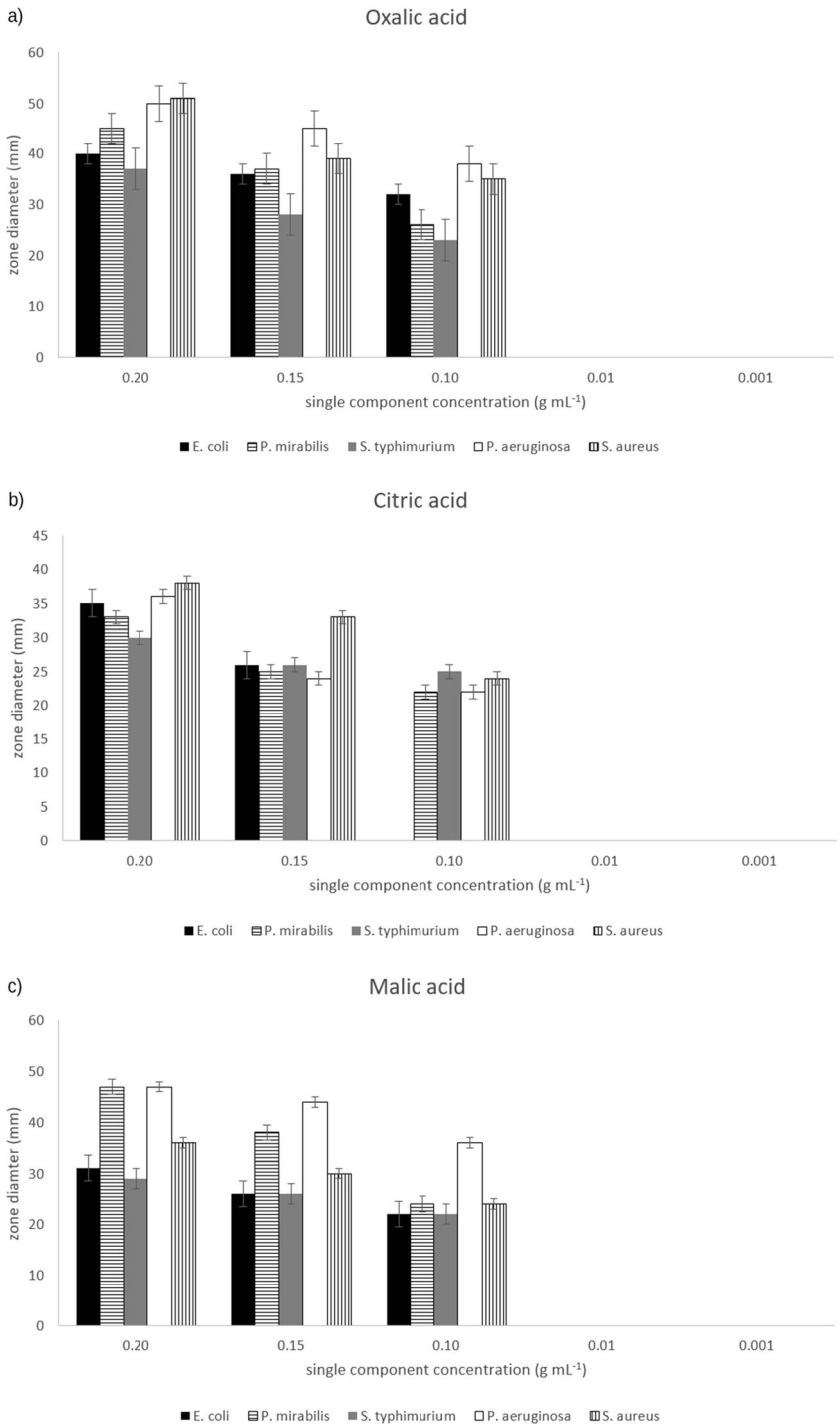

Fig. 1 Antimicrobial activity of oxalic, citric and malic acid to the most common pathogens

energy sources for cell growth. Glucose is mainly metabolised via glycolysis, providing energy and metabolic intermediates for the tricarboxylic acid pathway. Glucose also provides ribose by the pentose phosphate pathway, which is necessary for nucleic acid synthesis (Butler 2004). Xylitol and sorbitol, in addition to other sugars like glucose and fructose, can maintain the integrity of the carbohydrate requirement for cell growth, thus providing energy for cell metabolism and proliferation (Wang and van Eys 1981). Similar results were obtained for betaine NADES with glucose, malic acid and proline, which showed high cell tolerance (Table 3). Betaine, an intermediate metabolite of choline metabolism, is involved in protein and energy metabolism due to its methyl group donor function (Ratriyanto et al. 2009). Betaine is also known as intracellular osmolyte, which influences the osmolality of cell culture media. During cultivation, cell cultures are sensitive to osmolality variations, which can result in cell

Table 3 The EC_{50} values for HeLa, HEK293T and MCF-7 cells following exposure to NADES. Values are mean ($n = 3$) ± SD

NADES	HeLa cells		HEK293T cells		MCF-7 cells	
	EC_{50} (mg L^{-1})	EC_{50} (mM)	EC_{50} (mg L^{-1})	EC_{50} (mM)	EC_{50} (mg L^{-1})	EC_{50} (mM)
ChOx	330.90 ± 29.75	2.48 ± 0.27	> 2000	> 10	558.98 ± 54.32	4.19 ± 0.41
ChU	> 2000	> 10	> 2000	> 10	83.50 ± 7.94	0.81 ± 0.07
ChXylol	> 2000	> 10	> 2000	> 10	> 2000	> 10
ChSol	> 2000	> 10	> 2000	> 10	> 2000	> 10
BGlc	> 2000	> 10	> 2000	> 10	> 2000	> 10
BMaGlc	> 2000	> 10	> 2000	> 10	> 2000	> 10
BMaPro	*	*	*	*	*	*
CitPro	> 2000	> 10	> 2000	> 10	> 2000	> 10
CitGlcGly	> 2000	> 10	> 2000	> 10	> 2000	> 10
CitFruGly	> 2000	> 10	> 2000	> 10	> 2000	> 10

*Stimulatory proliferative effect

growth dropping (Butler 2004). Furthermore, betaine and proline are known osmoprotectants, which may attenuate the toxic effects of high osmolality on cell growth and have some overall positive effects on restoring cell growth and metabolic profile (Castilho et al. 2008). An interesting finding during this study was the concentration-dependent proliferative effect of BMaPro in all tested cells (data not shown). This observation could be explained by the synergistic effect of betaine and proline on the osmolality of culture media, obtained energy for cell growth from betaine and proline as a component for protein synthesis. Proline, as the amino acid component of cell culture media (and as a component of NADES as well), ensures the metabolic functions of cells since the absence of any of amino acid would trigger the onset of cell death (Ozturk and Hu 2006). Furthermore, it has been reported that NADES with organic acids, such as malonic acid, showed higher toxicity profiles in comparison to NADES with sugars (Hayyan et al. 2016). It seems that in this case, the toxic potential of malic acid in BMaPro is suppressed by two other components, betaine and proline, highlighting the tuneable properties of NADES.

Citric acid-based NADES with proline, glucose, fructose and glycerol did not have any cytotoxic effects on the tested cells (Table 3). Their effects are connected with the metabolic effects of glucose and fructose as energy sources, the role of glycerol in carbohydrate and lipid metabolism and the role of proline in the metabolism and osmolality of the culture medium. The obtained results are also in agreement with results reported by Hayyan et al. (2016), indicating the high tolerance of tested cells for carbohydrate NADES.

Evaluation of NADES antioxidant activity

The antioxidant activity of NADES is less known and understood, but nonetheless, several reports on this topic can be found (Hayyan et al. 2015; Nam et al. 2015; Radošević et al. 2016). According to the literature, the individual components of NADES, such as malic acid, citric acid, proline and betaine, possess antioxidative activity (Tang et al. 2013; Hakeem et al. 2014; Alirezaei et al. 2015). Therefore, the purpose of the conducted experiments was to find out if NADES with different compositions also have antioxidant activity. The antioxidant activity of the tested NADES was determined using the ORAC method (Fig. 2). The ORAC method is thought to be the most relevant method for determining antioxidant activity

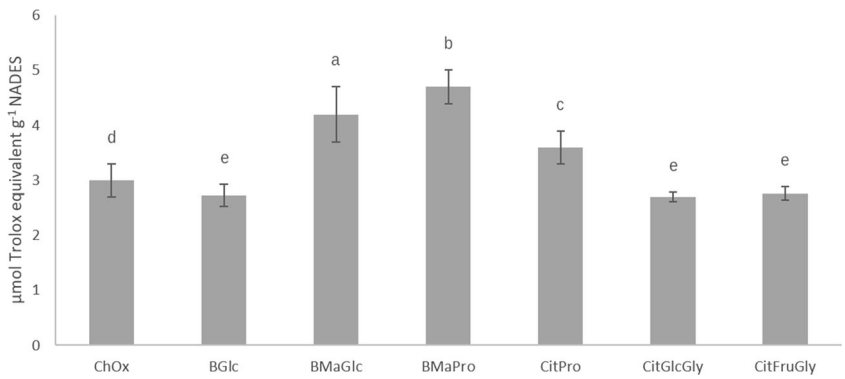

Fig. 2 The oxygen radical absorbance capacity (ORAC) of prepared NADES. Results are expressed as µmol TE g^{-1}. Mean values ± SD ($n = 3$) in each column followed by different lowercase letters are not significantly different ($p < 0.05$) as measured by Tukey's HSD test

because it utilises peroxyl radicals, which are a biologically relevant radical source, reflecting the major mechanisms of antioxidant action for evaluating relevance to cell protection (Ninfali et al. 2005; Karadag et al. 2009).

Among the prepared NADES, ChXylol, ChSol and ChU did not show antioxidative activity, which was not surprising since their forming compounds are not antioxidative, indicating that those NADES could not serve as radical scavengers. On the other hand, NADES that contained antioxidative compounds were also antioxidative. The ORAC values of other NADES were in the range from 0.7 to 2.7 μmol TE g^{-1} dw, with the highest values obtained for BMaPro, followed by BMaGlc > CitPro > ChOx > BGlc ≈ CitGlcGly ≈ CitFruGly.

Similarly, Hayyan et al. (2015) noted that the cholinum-based NADES with glycerine, ethylene glycol, triethylene glycol and urea as an HBD exhibit very low antioxidant activity. We observed the highest antioxidant activity for NADES comprising malic acid in contrast to those with citric acid, which corresponds to the different levels of antioxidative activity between these organic acids. The measured antioxidative activity of the forming compounds of NADES showed the highest antioxidative activity for malic acid among all tested compounds (data not shown). According to literature, malic acid is used as a food additive and has much higher degree of antioxidant activity than citric acid (Triantis et al. 2001). Furthermore, outside of antioxidative activity, NADES could have other biological activities since the compounds that compose NADES can possess various biological traits (Hayyan et al. 2015; Radošević et al. 2016). It has been proposed that NADES formed from compounds with proven pharmacological effects, such as an amino acid or an organic acid, could also have similar properties, indicating that not only could the physicochemical characteristics of solvents be fine-tuned but also their biological activity (Radošević et al. 2016). However, there is still not enough data on the antioxidant activity of NADES, which could be of interest for characterising and applying NADES properties.

Conclusions

Different bacteria and cell lines were observed to have different toxicity responses to the tested NADES. Most likely, the mechanism of NADES' action is associated with its interactions with cell membranes and demonstrated test system dependency. In this study, BMaPro showed a beneficial effect on the proliferation of tested cell lines as well as the highest antioxidant activity, which can be useful for optimising cell culture media for the protective and nutritional factors already presented in commercial formulations. Research whose primary purpose is the synthesis and application of NADES must be followed by an evaluation of their biological activity (e.g., antimicrobial activity, toxicity towards animal cell, antioxidative or other biological activity) to find the solvent with the best profile for wider industrial applications. Overall, our results indicate that it is not a good approach to consider NADES a priori as harmless solvents simply because most of their forming compounds are benign for humans and the environment. Due to the enormous chemical diversity and huge number of possible compositions of NADES, careful and critical assessment of the environmental impact to other trophic levels should also be performed.

Funding The work was supported through the Croatian Science Foundation HRZZ (Grant No. 9550).

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