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Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity

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ABSTRACT

In the present study phenolic grape skin extracts were prepared by using five choline chloride based natural deep eutectic solvents (NADESs) containing glucose, fructose, xylose, glycerol, malic acid and valorised by testing their biological activity *in vitro* using two human tumour cell lines (HeLa and MCF-7). Initially, used NADESs were investigated regard to their toxicity and low cytotoxicity of solvents was observed toward HeLa and MCF-7 cells (EC_{50} values > 2000 mg/L). Among used choline chloride based NADESs, the one containing malic acid showed the best performance concerning extraction efficiency (total phenolic and total anthocyanin were 91 and 24 mg/g dw), as well as antioxidant (ORAC values were 371 μmol TE/g dw) and antiproliferative activity (percentage of cell viability were about 20%). Herein, for the first time it was showed that NADES components could be chosen not only to fine-tune solvent physicochemical characteristics but also to enhance biological activity of extracts prepared in NADESs. Therefore, our research confirmed that NADESs are excellent and promising choice of solvents for sustainable and green extraction, which will lead to its novel application in food and pharmaceutical industry.

1. Introduction

A growing awareness of the human impact on the environment has pushed 'green extraction' as an alternative process with respect to scientific and industrial research and development. The demands for green extraction are based on the discovery and design of extraction processes offering an optimal utilisation of raw materials, solvents and energy (Chemat, Abert-Vian, & Cravotto, 2012). Also, one of the priorities put forward in the EU environmental policy and legislation for the 2010–2050 period is the decreased usage of petrochemical solvents and volatile organic compounds, since most of these solvents are flammable, volatile and often toxic. Accordingly, a growing area of research in the development of green extraction is devoted to designing new, environmentally friendly and tuneable solvents which would meet both technological and economic demands (Cvjetko Bubalo, Vidović, RadojčićRedovniković, & Jokić, 2015). Moreover, the manufacturers that use organic solvents in various processes have to fulfill rigorous safety demands, prove the absence of risk for workers during the extraction process and demonstrate safety of the final product regarding solvent traces.

Several types of solvents, including deep eutectic solvents (NADESs), have been suggested as candidates for green extraction (Cvjetko Bubalo et al., 2015). Representing a new generation of liquid salts, NADESs are generally based on mixtures of cheap and readily available components: non-toxic quaternary ammonium salts (e.g. cholinium chloride) and naturally-derived uncharged hydrogen-bond donors (HDB) (e.g. amines, sugars, alcohols and carboxylic acids). With respect to environmental and economic benefits, NADESs offer many advantages, including low costs, readily available components, simple preparation, a low toxicity profile and sustainability. These solvents feature very good physicochemical properties: negligible volatility, a liquid state even at temperatures far below 0 °C, adjustable viscosity, a wide polar range and a high degree of solubilisation strength for different compounds. Moreover, NADESs may be considered 'designer solvents' due to their numerous structural possibilities and the potential for designing their physico-chemical properties to accommodate different purposes. Ever since their emergence, NADESs have attracted attention as solvents in a variety of scientific

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and technological areas. In addition, NADESs are a class of solvents based on compounds that are safe for human consumption, a feature which promises great possibilities in the fields of drug-delivery systems, bone-therapy scaffolds and other pharmaceutical, cosmetic and food-related applications (Cvjetko Bubalo et al., 2015; Paiva et al., 2014). Several studies have reported successful NADES application in the extraction of phenolic compounds, indicating its great potential in plant-extract production for direct use in human consumption; however, a lack of data on toxicology and on the biological activity of the extract limits the commercial and industrial applications of NADES (Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013a; Dai, Witkamp, Verpoorte, & Choi, 2013b; Dai, Verpoorte, & Choi, 2014; Woo Nam, Zhao, Sang Lee, Hoon Jeong, & Lee, 2015).

On the other hand, grapes, one of most popular of fruits, are widely cultivated throughout the world. Recently, due to their health-promoting activity, grapes and grape products have attracted a great deal of interest among scientists and technologists alike (Teixeira et al., 2014; Xia, Deng, Guo, & Li, 2010). These health benefits arise mainly from the bioactivities of the grapes' phenolic compounds (e.g. anthocyanins, flavanols, flavonols, stilbenes and phenolic acids). There currently is a great body of evidence which supports the positive biological aspects of grapes, such as the fruit's antioxidant, antimicrobial, anti-inflammatory and anticancer properties, as well as the cardiovascular protection the fruit is suggested to provide (Teixeira et al., 2014; Vislocky & Fernandez, 2010; Xia et al., 2010). Therefore, in the last few years, the valorisation of grapes and their agro-industrial by-products as a source of biologically active compounds, together with their emergence as value-added products with potential applications in the pharmaceutical, food and cosmetic industries, have drawn a significant amount of attention (Teixeira et al., 2014). In order to produce high-quality extracts with valuable biological activities, conventional methods used in the extraction of grape phenolics should be improved. These conventional extraction processes are known to be quite laborious and time-consuming. For instance, conventional extraction of grape skin anthocyanins includes successive extraction with repeated solvent exchange (after 4, 12, 4 and 12 h) during 32 h of extraction (Lorrain, Chira, & Teissedre, 2011). Also, conventional extractions involve the use of large amounts of solvents, such as hydrocarbons, alcohols and chloro-alkanes, since the majority of phenolic compounds are not soluble in water (Ignat, Volf, & Popa, 2011). In some cases, such as an extraction of grape skin and seed proanthocyanidins, two steps extractions using first acetone/water followed by methanol/water are usually used (Ćurko et al., 2014). In addition, special attention should be paid to the choice of solvent in order to prevent target molecule degradation (Ignat et al., 2011; Revilla, Ryan, & Martín-Ortega, 1998).

Based on the discussion in the text above, the aim of the present study was to prepare various phenolic grape skin extracts by using NADESs and their valorisation by testing their biological activity in two human tumour cell lines (HeLa and MCF-7). Prior to their use as extraction solvents, the NADESs were also screened for their cytotoxicity in order to propose a truly eco-friendly extraction method for phenolic compounds from grape skin as a ready-to-use technology.

2. Materials and methods

2.1. Chemicals and materials

Methanol, acetic acid and Folin-Ciocalteau reagent (FC) were obtained from Merck (Darmstadt, Germany). Cholin chloride (ChCl), glucose, fructose, xylose, glycerol, malic acid, quercetin-3-*O*-glucoside, (+)-catechin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), and fluorescein (FL) were purchased from Sigma (St. Louis, MO, USA). Analytical standards of delphinidin-3-*O*-monoglucoside, cyanidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, peonidin-3-*O*-monoglucoside, and malvidin-3-*O*-monoglucoside were purchased from Polyphenols AS (Sandnes, Norway). Trypsin-EDTA (0.25%) and DMEM (Dulbecco's Modified Eagle Medium) were purchased from Gibco Invitrogen Corporation (Paisley, UK), while FBS (Fetal Bovine Serum) was purchased from Gibco Invitrogen Corporation (Auckland, New Zealand). WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} was purchased from Roche (Mannheim, Germany).

Grapes of the Croatian native red grape cultivar, *Vitis vinifera* cv. Plavac mali, originating from Dalmatia (Croatia southern vine-growing region) were harvested in their technological maturity in October 2012. The amount of 2 kg of randomly selected grapes was used for the study, where skins were immediately manually separated from the pulp, freeze-dried (Alpha 1-2 LD plus Christ, Germany) for three days at −40 °C and stored at −20 °C before analysis.

Two adherent human cancer cell lines obtained from the Ruđer Bošković Institute, (Zagreb, Croatia) were used in this work. MCF-7 cell line derived from breast adenocarcinoma (ATCC No. HTB-22) and HeLa cell line derived from cervical adenocarcinoma (ATCC No. CCL-2) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were maintained in T-flasks in the incubator with humidified atmosphere and 5% CO_2 at 37 °C, while individual experiments to test cytotoxicity of NADESs and biological activity of grape skin extracts were performed in 96-well plates.

2.2. Preparation of NADESs

Choline chloride (ChCl) and hydrogen bond donor (HDB) were dried in the vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA) at 60 °C for 24 h before use. The ChCl and HBD (glucose, fructose, xylose, glycerol, malic acid) at the respective molar ratio (Table 1) were directly weighed and the mixture was stirred in the sealed flask at 80 °C for 2–6 h until a homogeneous transparent colourless liquid was formed. NADES solutions with 30% of water were prepared from the starting solvent by adding the right amount of water in the respective weight ratio.

2.3. Cytotoxicity of NADESs

Prior to use for extraction purposes, NADESs were evaluated for their cytotoxicity by the WST-1 assay. Briefly, HeLa and MCF-7 cells were seeded in 96-well plates at a density of 5×10^4 cells *per* well in 100 μL of media. After overnight incubation, MCF-7 and HeLa cells were treated with tested compounds (ChFru, ChXyl, ChMa) in the nominal concentrations from 1 mg/L to 2000 mg/L. Following exposure for 72 h, WST-1 reagent was added to each well and cells were incubated for another 4 h, after which absorbance at 450 nm was measured on the micro plate reader (Tecan, Switzerland). Cell

Table 1
List of the natural deep eutectic solvents used in this study.

Combination	Abbreviation	Molar ratio
Choline chloride: glucose	ChGlc	2:1
Choline chloride: fructose	ChFru	1.9:1
Choline chloride: xylose	ChXyl	2:1
Choline chloride: glycerol	ChGly	1:2
Choline chloride: malic acid	ChMa	1:1