Green technology meets ecotoxicology

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Summary

By applying concept and principles of green chemistry into different technological processes, green technologies are developed. The environmental and economic benefits of "green" approach is achieved through several directions, such as the use of renewable raw materials, creation of economic efficiency, the use of alternative reaction conditions, as well as the application of non-conventional solvents. From the point view of green chemistry, alternative solvents, in order to be a "green" substitution to hazardous organic solvents, should be: non-volatile, non-flammable, stabile, synthesized by an environmentally friendly procedure, nontoxic and biodegradable. The toxic impact of all newly synthesized chemicals, such as alternative solvents, could be determined by methods and techniques of ecotoxicology. Ecotoxicology, an interdisciplinary scientific field, can serve as a way of monitoring the greenness of the processes. *In vivo* and *in vitro* experiments are used to study the effects of chemicals on different levels of organizations, from molecules to communities and ecosystem. The usage of *in vitro* methods is encouraged by a scientific community and regulatory agencies as an alternative to *in vivo* studies in order to reduce the number of laboratory animals used in the toxicological studies. Therefore, in this paper we gave a brief overview on the usage of animal cell cultures within the field of green chemistry and technology.

Keywords: cell lines, deep eutectic solvents, ecotoxicology, green technology, ionic liquids

Introduction

The story about green technologies starts with green chemistry, a relatively new scientific field which was first defined in the year 1991 by Paul Anastas and John Warner in their book "*Green Chemistry: Theory and Practice"*. They defined green chemistry as "*The way of creation and application of chemical products and processes that reduce or eliminate the use or production of substances hazardous to human health and the environment.*", what is rather similar to the newer definition by US EPA (2012) which says that green chemistry is: "*Design of chemical products and processes that reduce or completely remove the application and creation of harmful and dangerous substances.*" The definition of green chemistry is rather clear and simple, but the its whole concept is more complex and is summarized in the twelve principles of green chemistry (Table 1). By following those principles and satisfying most of these criteria in the technological process of interest, the green technology is implemented.

Table 1. The twelve principles of green chemistry (adopted from Anastas and Warner, 1998)

1) Prevention It is better to prevent waste than to treat or clean up waste after it has been created.	**2) Atom Economy** Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.	**3) Less Hazardous Chemical Synthesis** Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to people or the environment.
4) Designing Safer Chemicals Chemical products should be designed to effect their desired function while minimizing their toxicity.	**5) Safer Solvents and Auxiliaries** The use of auxiliary substances (e.g., solvents or separation agents) should be made unnecessary whenever possible and innocuous when used.	**6) Design for Energy Efficiency** Energy requirements of chemical processes should be recognised for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7) Use of Renewable Feedstocks A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.	**8) Reduce Derivatives** Unnecessary derivatization (use of blocking groups, protectiodde-protection, and temporary modification of physicalchemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.	**9) Catalysis** Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10) Design for Degradation Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.	**11) Real-time Analysis for Pollution Prevention** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.	**12) Inherently Safer Chemistry for Accident Prevention** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

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The goal of green chemistry, which is to obtain the environmental and economic benefits, is achieved through several directions such as the biocatalysis, the use of renewable raw materials, creation of economic efficiency, the use of alternative reaction conditions (ultrasound- and microwave-assisted processes), as well as the application of non-conventional solvents as a substitute to hazardous organic solvents. From the point of view of green chemistry, solvents should be: non-volatile, in order to reduce air pollution; non-flammable, for process safety; stabile, for recycling and reusing potential; synthesized by an environmentally friendly synthetic procedure; nontoxic and biodegradable, for environmental impact (Matzke et al., 2010). The enumerated criteria were partially fulfilled with ionic liquids (ILs), and later with deep eutectic solvents (DESs). The development of ILs started a few decades ago and they were considered as a promising alternative to traditional organic solvents, from both the environmental and technological perspectives. In the recent years, the perception of their "greenness" was partially changed, as the scientific community intensively assessed the risk of their application based on the entire lifecycle, including preparation methods, methods of their degradation after use and their impacts on the ecosystem. Also, the limitations of conventional ILs are high cost (5-20 times higher than the cost of conventional organic solvents), toxicity, similar to or even higher than organic solvents, and generally poor biodegradability (Cvjetko Bubalo et al., 2014). Due to the above mentioned drawbacks, scientific researches went further on with the investigation of alternative solvents and the 4th generation of ILs entered the stage. The 4th generation is, namely, natural ILs and deep eutectic solvents, which have in common that they can be synthesized from natural or renewable compounds. Although DESs are sometimes referred as 4th generation of ILs, it is not correct, since they are not composed of ions. Actually, DESs are prepared by mixing two or three low-cost components (e.g. quaternary ammonium salts, amides, organic acids, polyalcohol), forming a eutectic mixture based on hydrogen bonding interactions. DESs have similar physical properties and phase behaviour to ILs and retain most of their excellent technological properties, but at the same time being low-cost alternative with the presumed better environmental profile (Hou et al., 2013b; Hayyan et al, 2013a; Hayyan et al, 2013b). Overall, investigations on alternative solvents are directed towards three major domains: 1) synthesis and physicochemical characterization, 2) evaluation of environmental impact and 3) possible applications in green processes and technologies (Fig. 1).

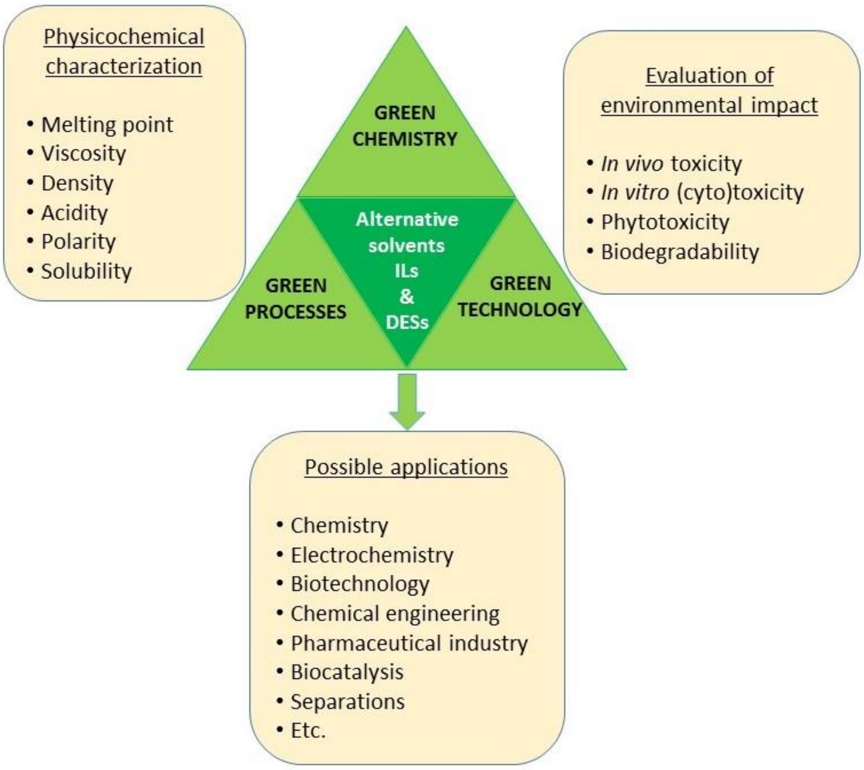

Fig. 1. Schematic presentation of three major directions in investigations on alternative solvents

Ecotoxicology, an interdisciplinary scientific field based on the combined knowledge of ecology and toxicology, can serve as a way of monitoring the greenness of the process. By applying methods and techniques of ecotoxicology we can check the twelve principles of green chemistry in order to elucidate if the obtained process and/or technology satisfies most of those criteria and whether it is or it is not in agreement with the concept of green chemistry. The toxic impact of newly synthesized chemicals, such as ILs or DESs, or those already present in the environment could be determined *in vivo* or *in vitro*. *In vivo* experiments are necessary for the final conclusion on toxicity with respect to humans and environment, but those experiments are unethical due to the usage of laboratory animals, are time-consuming and expensive. Therefore, scientific community, as well as regulatory agencies encourage the usage of alternative *in vitro* methods as they are faster, cheaper and easier to standardize. Consequently, scientific laboratories follow the principle of the 3Rs to "Reduce, Refine and Replace" the use of animals in the laboratory research. Also, recent EU Regulation, so called REACH (EC1907/2006; Registration, Evaluation, Authorization and Restriction of Chemical substances), emphasizes the use of alternative *in vitro* models and work on the development, validation and regulatory acceptance of alternative tests with a goal of reducing the number of laboratory animals used in the toxicological studies.

The use of animal cell cultures for assessment of alternative solvents

The aim of ecotoxicology is to study the effects of chemicals on different organisms at the population, community or ecosystem level. Studies on population, community or ecosystem level have high ecological relevance, but they are time-consuming and hard to standardize (Fig. 2). Usually, they give insight into the chronic effect and could provide information on multigenerational impact. Studies on organisms and lower levels of organization (tissues, cells, and biomolecules) are easy to standardize and provide data on the acute effects and the possible mechanism of action of the investigated chemical. When talking about investigations from organisms to biomolecules, we distinguish *in vivo* and *in vitro* studies, both having their pros and cons. In this paper we gave insight into the usage of animal cell cultures, as an alternative to *in vivo* tests on living organisms, within the field of green technology.

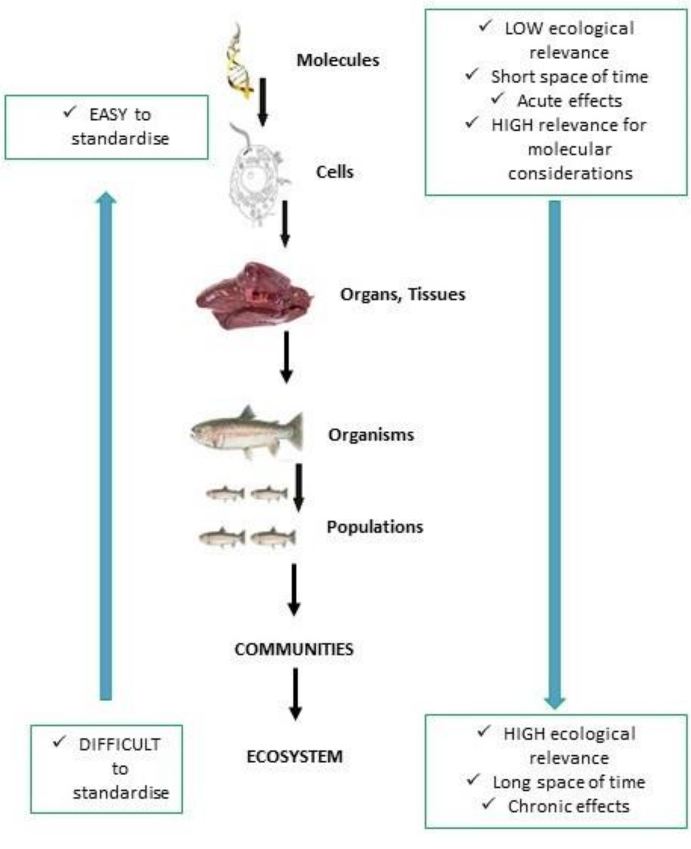

Fig. 2. Ecotoxicological concept – from molecular to ecosystem studies

Ecotoxicology techniques and methods could be applied as a way of monitoring the green processes by checking the principles 3, 4, 5, 10 (Table 1). Principle no. 3 – "Less hazardous chemical synthesis", means that all reactants, auxiliary chemicals, and solvents used during process, as well as the final product, should be non-toxic to humans and environment, if possible. Principle no. 5 - "Safer solvent and auxiliaries" includes development of solvent alternatives to organic hazardous solvents. In order to prove that those alternative solvents are truly green, methods of ecotoxicology have to be applied. That principle is related to the principle no. 4 - "Designing safer chemicals", where the usage of QSAR (Quantitive Structure-Activity Relations) methodology, as one of the ecotoxicological methods, could be applied to mathematically predict the impact of the structure on the toxicity of a compound. Finally, principle no. 10 - "Design for Degradation", is a question of (bio)degradation, which is also under the scope of ecotocixological field.

Our work on solvent alternatives to organic hazardous solvents started with the development of ILs, organic salts consisting entirely of ions with melting points lower than 100 °C. Regarding the structure of ILs, cations are usually variously substituted bulky organic molecules of low symmetry containing a positively charged nitrogen, sulphur or a phosphorus atom, while typical anions are usually inorganic or organic species such as halides, tetrafluorborate, hexafluorphosphate, bis(trifluoromethylsulfonyl)imide, acetate and dicyanamide (Earle and Seddon, 2000). The number of possible combinations of different cations and anions is enormous and it is estimated that roughly 10^{18} ILs are possible. This fact ensures tuning of their chemical and physical properties (e.g. melting point, solubility, acidity, hydrophobicity, density and viscosity) in order to design an optimal IL for specific applications (Pham et al., 2010), which can be seen in Fig. 1. Similar to "classical" ILs, natural ILs and DESs could also be designed for the required application. Besides chemical and physical properties, environmental impact of ILs also differs depending on their structure.

Overall, toxicity studies on ILs were carried out by performing a series of tests on bacteria, yeast, multicellular organisms (such as nematode, water snail, water flea, zebra fish green seaweed), soil invertebrates (such as spring tail, duckweed, watercress, spring barley, wheat), mammals, as well as on different mammalian cell lines (Cvjetko Bubalo et al., 2014). *In vivo* toxicity of ILs is classified according to Passino and Smith (1987) or by UFT Merck Ionic Liquids Biological Effects Database (http://www.il-eco.uft.uni-bremen.de), which also defines *in vitro* classification. The latter database is very good and comprehensive since more than 300 different ILs and their precursors using an (eco)toxicological test battery of different biological complexity have been investigated. Furthermore, several biodegradation tests were performed to determine the biodegradability of IL structures. In that database ILs toxicity data are presented as EC_{50} values for enzyme (acetylcholinesterase) inhibition; inhibition of bioluminescence of marine bacteria *Vibrio fischeri*, the growth inhibition of algea *Lemna minor* and higher plant *Scenedesmus vacuolatus* and the viability of IPC-81 cell line (Fig. 3).

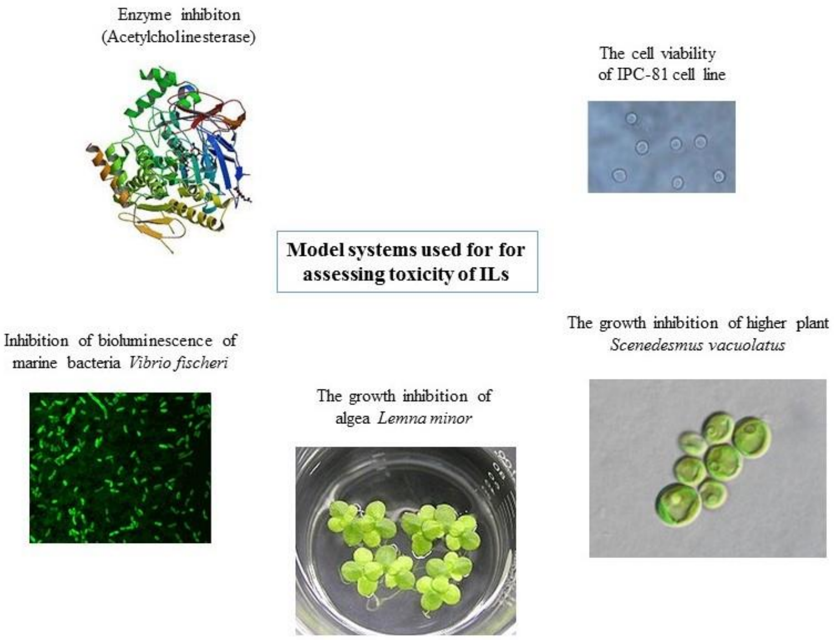

Fig. 3. Model systems used by UFT Merck Ionic Liquids Biological Effects Database for assessing toxicity of ILs

In general, depending on the used model system, good correlations between *in vivo* and *in vitro* results for toxicity testing of ILs were observed. Toxicity was structure-dependent, with the type of cation and the type and length of alkyl substituents bonded to a heterocyclic ring, being the most influential on the outcome of the toxicity study (Cvjetko Bubalo et al., 2014; Matzke et al., 2007; Radošević et al., 2013; Ranke et al., 2007). Our group contributed to the field of ILs toxicity with the assessment of cytotoxicity of imidazolium ILs on the fish cell line and the comparison of the obtained results with human cell lines, which were frequently used by other authors. In our first work by Cvjetko et al. (2012) the cytotoxicity of imidazolium ILs toward ovarian fish cell line CCO (*Channel Catfish Ovary*) and the human tumour cell line HeLa was evaluated by the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell viability assay and a good correlation was obtained. Such result encouraged us to continue with assessing cytotoxicity of ILs on CCO cells in order to determine whether used fish cell line can serve as an alternative to *in vivo* test on fishes. Because of low vapour pressure of ILs, air pollution is not expected, but the risk for aquatic environment is possible in case of a bigger industrial production and accidental spills. In the aquatic risk assessment of ILs acute *in vivo* toxicity tests on *Zebra fish* and *Gold fish* are reported as well as *in vivo* inhibition assays using different aquatic species such as *Daphnia magna*, some algae and marine bacteria *Vibrio fischeri*. Therefore, we compared our results presented in paper by Radošević et al. (2013) with those of Samori et al. (2010) and Pretti et al. (2006) and concluded that the fish cell line could be a good biological system for initial toxicity testing of ILs that could replace *in vivo* bioassays, since a good correlation of *in vitro* and *in vivo* results was determined. Furthermore, by assessing the cytotoxicity of imidazolium ILs containing different anions and alkyl chain lengths, as a substituent at the cation ring, and by applying fluorescent microscopy and flow cytometry, we showed concentration and structure dependent cytotoxicity of tested ILs in CCO cells. Cytotoxicity was related to the type of anion and alkyl chain length, with EC_{50} values ranging from low to high cytotoxicity, what is probably related to a membrane disruption and necrosis (Radošević et al., 2013). The number of papers on the toxicity of ILs increased exponentially during the last 2 decades, during which the perception of ILs as eco-friendly solvents was changed, but altogether, obtained data and knowledge resulted in general guidelines for the synthesis of “greener“ ILs (Cvjetko Bubalo et al., 2014). By following those guidelines, we can synthesize ILs which are truly environmentally friendly while still retaining their excellent application possibilities. Actually, having enough knowledge on structure-effect relations, makes it possible to select cations and anions necessary to synthesize ILs with the desired physicochemical properties for planned application, which is seen as a real advantage and power of these alternative solvents often called “designer“ solvents.

As already mentioned, natural ILs and DESs are in the spotlight of a scientific community over the last five years, due to the assumption that they are more favourable for the environment. Most of the work on toxicity of natural ILs and DESs was done on enzymes, representative bacteria, the marine bacterium, the crustacean and a few cell lines (Hou et al., 2013a; Gouveia et al., 2014; Ventura et al., 2014). There is still insufficient data about their toxicity, so our team also continued investigating those alternative solvents. In a work by Radošević et al. (2015) three choline chloride-based DESs with glucose, glycerol, and oxalic acid as hydrogen bond donors were evaluated for *in vitro* toxicity using fish and human cell line, phytotoxicity using wheat, and biodegradability using waste water microorganisms through the closed bottle test. The obtained data indicate that the three studied ChCl-based DESs possess low to moderate cytotoxicity, do not inhibit wheat seed germination and are classified as “readily biodegradable“. A good correlation between toxicity and biodegradability was found, suggesting that ChCl-based DESs have a “green“ profile and a good prospect for a wider use. Furthermore, we conducted comparative *in vitro* study with respect to fish cell line and concluded that cholinium-based ILs and DESs (Radošević et al., 2016b) have a better ecotoxicological profile than classical ILs (Fig. 4) and should be therefore utilised in the development of alternative solvents.

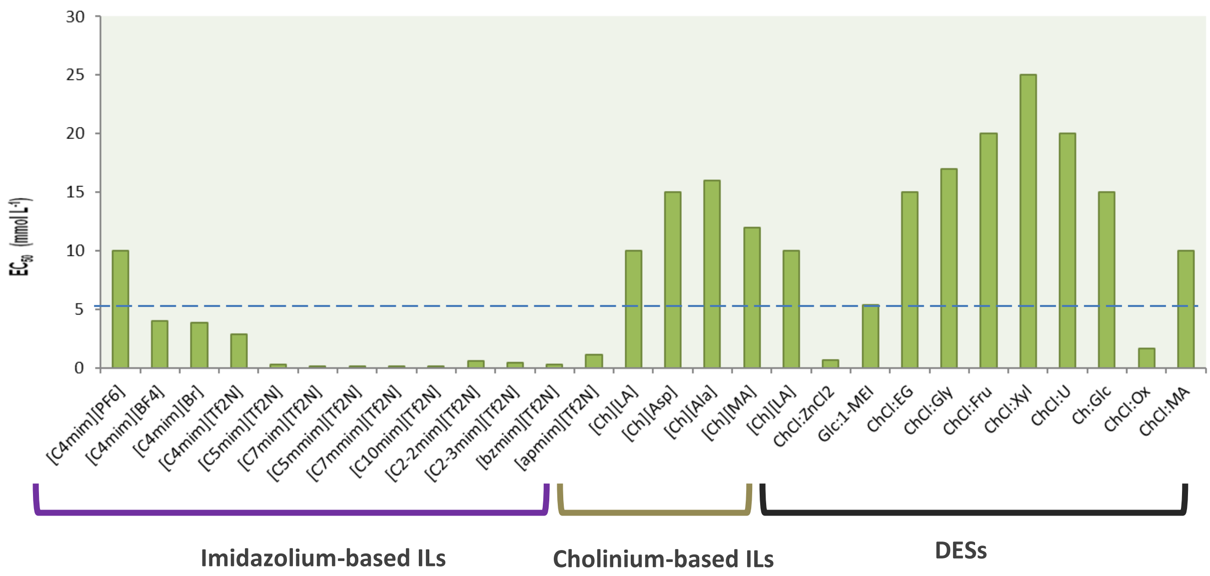

Fig. 4. Graphical comparison of EC_{50} values obtained for imidazolium-based ILs, cholinium-based ILs and DESs with respect to CCO cell line (Cvjetko et al., 2012; Radošević et al., 2013.; Radošević et al., 2015; Cvjetko Bubalo et al., 2015; Radošević et al., 2016b). Dashed line represents a border-line between moderate and low cytotoxicity (EC_{50}>5 mM) according to UFT Merck Ionic Liquids Biological Effects Database.

Even though numerous applications of alternative solvents are expected, currently they are not used for commercial purposes. Only some companies have started with the industrial use of ILs, for example the French Petroleum Institute (Olivier, 1999) in Difasol process, and BASF's in BASIL process, which was the first large-scale industrial application of ILs (Masse and Massonne, 2005). To the current knowledge, there are no data about the presence of those solvents in the environment, although rising interest for these compounds and a great number of possible applications will surely result in their bigger industrial use and consequently in an uncontrolled transfer in the environment. Therefore, environmental impact and fate (e.g. biodegradation and ecotoxicity) of ILs and DESs has to be extensively and critically evaluated before their large-scale production.

Based on the current scientific knowledge on the alternative solvents, our expertise, and references in this field, we can conclude that alternative solvents, ILs and DESs, will surely be solvents of the future. Further investigation on ecotoxicological profile of alternative solvents is still needed, but one should bear in mind that although certain solvents possess moderate toxicity, which is undesirable from the aspect of green chemistry, their physicochemical properties may be beneficial for the desired application and should therefore not be neglected. For example, ChCl-based DESs with organic acids proved to be most inhibiting for bacteria and cell growth (Paiva et al. 2014; Radošević et al. 2015), but on the other hand, acidity of those DESs is good for their application as solvents for the extraction of biologically active compounds from plants (Dai et al., 2013a; Dai et al, 2013b). Also, those ILs and DESs which showed antimicrobial activity (Hayyan et al., 2013a; Hayyan et al., 2013b), and/or inhibited growth of human tumour cell lines (Hayyan et al., 2015), could be considered as compounds with a biological activity and used in the development of antimicrobials and other pharmaceuticals. Taken all together, the alternative solvents, which will satisfy technological, environmental and economic criteria, will be surely applied in different kinds of technologies and industries in the future.

The use of animal cell cultures for assessment of extracts obtained by alternative solvents with possible application in food industry

Human cell lines are already widely used in testing biological activity of compounds present in plants and plant’s products. The application of *in vitro* cell cultures for this purpose is based on the fact that interaction of the test substance and biological systems occurs preferentially at the cellular level, and has an impact on the whole organism (Ekwall, 1995). With such approach a large number of samples, including crude extracts from screening phase, as well as pure fractions obtained during the isolation procedure, could be evaluated. Beyond that, the application of *in vitro* cell cultures is a good choice for preliminary screen of biological activities, which enables faster and more cost-effective studies compared to *in vivo* studies. The findings obtained by *in vitro* tests are surely valuable and can serve as guidelines for further *in vivo* and epidemiological studies of biological activity of plants, its compounds, and products.

Apart from ecotoxicological investigations of alternative solvents, products obtained by those solvents could be also assessed for cytotoxicity using animal cell cultures. One of the possible applications of the alternative solvents is extraction. Lately "green extraction" emerged as an alternative to the classical extraction with organic solvents. There are several reports about successful application of the natural deep eutectic solvents (NADESs) for the extraction of phenolic compounds (Dai et al., 2013a; Dai et al, 2013b; Woo Nam et al, 2015, Radošević et al., 2016a), which indicates their great potential in the production of plant extracts. Since NADESs are a class of solvents based on compounds that are safe for human consumption, their application as the extraction solvents has great possibilities for the direct use of such extracts in human consumption. Although NADES consist of naturally occurring compounds with a presumed high safety profile, possible synergetic effect between components may result in a toxicological profile different than expected and, therefore, toxicity of NADES, as well as the biological activity of the extract, must be further investigated to prove that such application of NADES is safe for the commercial and industrial usage.

The same *in vitro* cytotoxicity approach used for the assessment of alternative solvents can be applied for the assessment of extracts obtained by the same solvents. In our work by Radošević et al. (2016a) various phenolic grape skin extracts were prepared in NADESs and tested with regard to their biological activity on two human tumour cell lines (HeLa and MCF-7). Prior to their use as the extraction solvents, the NADESs were also screened for their cytotoxicity. The tested NADESs (choline chloride: glucose, choline chloride: fructose, choline chloride: xylose, choline chloride: glycerol and choline chloride: malic acid) possessed low cytotoxicity and were, therefore, good candidates for the green extraction of grape skin phenolics. Four out of five obtained extracts had a better cytotoxic potential with respect to HeLa and MCF-7 cells than a classic methanol extract, with choline chloride: malic acid being the most pronounced. Also, a high positive correlation between the content of phenolic compound, ORAC value for antioxidative capacity and cytotoxicity with respect to cancer cells was obtained, indicating a potential anticancer activity of such grape extracts. With such a comprehensive approach, it is possible to propose a truly eco-friendly method for the extraction of phenolic compounds from grape skin and maybe develop it as a ready-to-use technology.

Conclusions

In order to assess that alternative solvents and products obtained by those solvents are truly green, methods and techniques of ecotoxicology could be applied. *In vitro* methods as faster, cheaper and easier to standardize are proved to be a good alternative to *in vivo* studies and can surely serve as valuable guidelines for further *in vivo* and epidemiological studies. Current literature data on ILs and DESs as well as plant extracts produced by those solvents confirm the value of animal cell cultures used for determination of their *in vitro* cytotoxicity and show that ecotoxicological methods and techniques should be used to monitor and evaluate green processes and technologies.

Acknowledgements

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