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GREEN EXTRACTION OF ANTIOXIDANTS FROM NATURAL SOURCES WITH NATURAL DEEP EUTECTIC SOLVENTS

Hristo Petkov, Boryana Trusheva, Slaveya Krustanova, Tsvetinka Grozdanova, Milena Popova, Kalina Alipieva, Vassya Bankova

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Abstract

Natural deep eutectic solvents (NADES) have been developed in order to replace traditional volatile and toxic organic solvents for the extraction of biologically active substances from natural sources. Recent research has shown that NADES are able to retain and even improve the antioxidant properties of the molecules dissolved in them. The aim of the present study was to evaluate the antioxidant activity of the extracts of two medicinal plants, *Plantago major* and *Sideritis scardica*, and of propolis with 10 different NADES. The results obtained confirm that NADES extracts of medicinal plants and propolis have a good antioxidant potential, allowing obtaining natural antioxidants without the application of organic solvents. Because of the biocompatibility and low toxicity of the NADES, the opportunity to apply these extracts directly in formulations, such as cosmetics, food supplements, etc., seems promising.

Key words: natural deep eutectic solvents, *Sideritis scardica*, *Plantago major*, propolis, antioxidant activity

Introduction. The extraction of biologically active substances from natural sources (medicinal plants, bee products) was one of the first processes that attracted the attention of researchers in the field of green chemistry. Finding "green" solvents to extract these substances, in order to replace traditional volatile and

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toxic organic solvents, is imperative due to the growing market for phytoproducts – medicines, food supplements, cosmetics, etc. [1]. As a result, a new kind of "green" solvents, the deep eutectic solvents (DESs), have been developed. A deep eutectic solvent is obtained from solids which, when mixed in a strictly defined ratio, form a eutectic mixture [2]. The formation of DES is explained by the formation of intermolecular hydrogen bonds between the molecules of the components – hydrogen bond donors and hydrogen bond acceptors. Consequently, the melting point of the mixture is lower than the melting points of the starting materials, and it is liquid at ambient temperature [3]. Combinations of natural substances (primary metabolites) in the solid state can also form such eutectic mixtures, liquid at ambient temperature – natural deep eutectic solvents (NADES) [4]. NADES have low vapour pressure, which is a significant advantage in terms of protecting the environment and human health, but makes it difficult to obtain solutes by removing the solvent. However, recent research has shown that NADES are able to retain and even improve the antioxidant properties of the molecules dissolved in them [5,6]. The application of NADES for the extraction of phenolic compounds from plants, which are usually active antioxidants, has shown promising results [7,8]. NADES are biodegradable and biocompatible, so there are no obstacles to their use, bypassing the solvent removal problem. The aim of the present study was to evaluate the antioxidant activity of the extracts of two medicinal plants, *Plantago major* and *Sideritis scardica,* and propolis with different NADES and to reveal the perspectives of the use of such extracts in cosmetics, food additives and medical devices.

Materials and methods. ***Plant material and propolis.*** Aerial parts of the studied plants were used. *Plantago major* was collected in March 2018 in the valley of Struma river, Bulgaria, a voucher specimen (No SOM 1390) has been deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences (IBER-BAS). *Sideritis scardica* collected in June 2017, was cultivated in the Western Rhodope Mountain, a voucher specimen has been deposited in the Herbarium of the IBER-BAS, No SOM 1391. Plant material was collected and identified by Assoc. Prof. Dr. Ina Aneva. Propolis was collected in 2018 in the area of the town of Elena, Balkan Mountains, Bulgaria.

Preparation of the NADESs. Ten natural deep eutectic solvents were prepared and tested (Table 1). For their preparation two methods were used [11]. CiA:PrD 1:4, XX:Gly 1:2, Bet:MA:H_2O 1:1:6, LA:Fr 5:1, LA:PrD 1:1, XX:PrD 1:3, XX:PrD:H_2O 1:1:1 and XX:Glu:H_2O 5:2:25 were prepared by mixing the components and subsequently stirring in a water bath (300 rpm) combined with mild heating at 50 °C until a homogeneous liquid was formed. XX:Ur 1:1 and XX:Xy 4:1 were prepared by vacuum evaporating method as the components were dissolved in water.

Polarity measurements. The polarity of the solvents was measured using the solvatochromic dye Nile red [12]. The dye was dissolved in the respective

Table 1

Composition and characteristics of the solvents

Abbreviation	Component 1	Component 2	Molar ratio	Water (molar parts)	E_{NR} [kcal/mol]	Density [g/mL]
BMAH	Betaine	Malic acid	1:1	6	48.87	1.25
CAPD*	Citric acid	1,2-Propanediol	1:4	0	48.87	1.19
LAFr	Lactic acid	Fructose	5:1	0	47.97	1.28
LAPD	Lactic acid	1,2-Propanediol	1:1	0	49.59	1.11
XXGlH*	Choline chloride	Glucose	5:2	25	49.17	1.16
XXGly*	Choline chloride	Glycerol	1:2	0	50.03	1.17
XXPD**	Choline chloride	1,2-Propanediol	1:3	0	50.69	1.06
XXPDH	Choline chloride	1,2-Propanediol	1:1	1	50.29	1.07
XXU*	Choline chloride	Urea	1:1	0	50.12	1.09
XXXy*	Choline chloride	Xylitol	4:1	0	49.90	1.15
EtOH 70%					50.87	0.88

*Data from [9]; **Data from [10]

solvent in the concentration range 0.01–0.1 mM and the absorption spectra were recorded on a Thermo Scientific Helios Gamma UV-vis spectrophotometer at room temperature. The λ_{max} was determined and used to calculate the molar transition energy E_{NR}, based on the equation: $E_{NR} = hcN_A/\lambda_{max} = 28591/\lambda_{max}$, where E_{NR} is in kcal.mol^{-1} and λ_{max} is in nm.

Density measurements. The NADESs density was determined as follows: 2 mL of NADES were put in a volumetric flask at 20 °C and the weight of the liquid was measured. The density was calculated using the formula: $\rho = m_{NADES}/V_{NADES}$, where ρ is density, g/mL at 20 °C, m_{NADES} is weight, g at 20 °C, and V_{NADES} is volume in mL at 20 °C (2 mL). For each solvent the procedure was performed in duplicate.

Extraction. Propolis was ground after freezing, air-dried plant material was ground using a coffee mill. The extraction of the ground material was performed in a 2 mL Eppendorf tube with 50 mg of propolis or plant material and 1.5 mL solvent in an ultrasound bath (ElmasonicS 30 H), without heating, for 1 h. The mixture was then centrifuged at 13 000 rpm for 40 min and filtered through cotton in a 1 mL volumetric flask. These extracts were further analyzed to determine the main groups of bioactive compounds, and to evaluate their antioxidant activity. Each extraction procedure was performed in triplicate.

Quantitative determination of total phenolics and total flavonoids. For measuring those two groups of bioactive compounds in studied NADES extracts we used the previously reported methods, described in [13]. For blank: solution of respective NADES instead of test sample (NADES extract) was used in analogous procedures. Every assay was carried out in triplicate. For propolis,

total phenolics content was estimated using calibration curve of standard mixture pinocembrin-galangin 2:1, and for total flavonoids – using a calibration curve of galangin; for the plant extracts, total phenolics content was estimated using caffeic acid as standard, and total flavonoid content with rutine as standard.

DPPH test. The scavenging activity of the extracts against DPPH free radical (RSA) was determined according to a previously described procedure [14]. Propolis extract, 0.5 mL, was diluted to 25 mL with 70% MeOH. Two millilitres of fresh methanolic DPPH solution (0.1 mM) was mixed with 100 µL aliquot of each tested sample. For the plant extracts, 1 mL plant extract was diluted to 10 mL with 70% MeOH; 100 µL aliquot was mixed with 2 mL DPPH methanolic solution. Every sample was analyzed in triplicate.

The decrease of the absorption was measured after 30 min storage in a dark place at 517 nm using UV-vis spectrophotometer Thermo Scientific Helios gamma.

The results were expressed as percentages with respect to a control value. The radical scavenging activity of the tested samples was calculated by the following equation:

$$\text{RSA } (\%) = [(A_0 - A_s)/A_0] \times 100,$$

where A_0 is the absorbance of the control sample (to which 100 µL 70% MeOH were added instead of aliquot volume of a sample), and A_s is the absorbance of the tested sample.

FRAP test. This sensitive and simple technique is commonly used to evaluate the antioxidant capacity of biological fluids, plant extracts and pure compounds. In its essence, ferric ion complexed to 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) acts both as an oxidant and a chromophore. The reaction of the Fe^{3+}-$(TPTZ)_2$ complex with an antioxidant generates the reduced form Fe^{2+}-$(TPTZ)_2$ that absorbs light at around 600 nm [15].

The assay was performed according to [16] with slight modification. The FRAP reagent was freshly prepared by mixing ten parts of 0.3 M acetate buffer (pH 3.6), one part of TPTZ in 40 mM HCl and one part of 20 mM $FeCl_3.6H_2O$ in distilled H_2O. The reaction was started by mixing 3 mL FRAP reagent with 100 µL of the investigated sample (diluted with 70% MeOH, as in the DPPH test). The reaction time was 30 min at room temperature in darkness and the absorbance was measured at 593 nm against a blank. The FRAP value was calculated from a calibration curve of $FeSO_4.7H_2O$ standard solutions and expressed as µmol Fe^{2+}/L.

Results and discussion. The choice of the ten NADESs used for extraction, listed in Table 1, was based on literature data on their polarity. As a reference solvent, 70% ethanol was applied. In our study, we determined the polarity and density of the studied NADES experimentally (Table 1). Because of the high viscosity of NADES, ultrasound assisted extraction was used in order to accelerate the extraction process.

The effectiveness of the extraction was evaluated by the percentage of extracted total phenolics and total flavonoids; part of these data has already been published, as noted in Tables 2 and 3. The antioxidant activity of the extracts was assessed using DPPH free radical-scavenging ability and ferric reducing antioxidant power (FRAP) assays. The results for *S. scardica* and *P. major* extracts are presented in Table 2; for propolis in Table 3. The 70% ethanol extracts of the plants and propolis were analyzed for their antioxidant potential, as a reference.

From the obtained results it is obvious that in some cases the extraction with NADES is more productive than the one with 70% EtOH, especially for phenolic compounds.The best yields were obtained with the solvent XX:Glu:H_2O, 5:2:25 for both plants; for *S. scardica* the value of total phenolics was 62.70 ± 1.50 mg CAE/g, and of total flavonoids 12.00 ± 0.65 mgRE/g. This NADES demonstrated also the best antioxidant activity in DPPH and FRAP tests, for *P. major* the %RSA was even higher than the one of the ethanol extract.

In general, NADES were more effective for the extraction of total phenolics than for flavonoids: the percentage of extracted flavonoids was lower in all solvents, compared to 70% ehtanol.

Propolis is a matrix very different from plant material, and its specific composition requires the application of different, specific calibration standards for the quantification of total phenolics and total flavonoids. The results of the extraction of propolis with the tested NADES and the antioxidant activity of the extracts are presented in Table 3. Expectedly, the most effective extractants for propolis turned out to be different from those most effective for *S. scardica* and *P. major*. The NADES XX:PrD:H_2O 1:1:1; XX:PrD 1:3, and LA:PrD 1:1 extracted higher amounts of phenolics and practically the same amounts of flavonoids, compared to 70% ethanol. The different nature of propolis constituents, and especially the lack of flavonoid glycosides and other glycosylated phenolics (such as phenylethanoilds), could be the reason for these differences. The extracts with the XX:PrD 1:3, and LA:PrD 1:1 solvents were also most active against DPPH radicals, similar to the ethanol extract. In the case of FRAP, however, none of the NADES extracts had capacity comparable to the ethanol extract of propolis, LA:PrD 1:1 being the most potent one but yet of lower activity.

In general, there was a strong positive correlation between the total phenolic content of the propolis extracts and the antioxidant potential: correlation coefficient for the RSA values was $r = 0.75$ ($p < 0.01$), and $r = 0.84$ ($p < 0.01$) for the FRAP values. The correlation between total flavonoids and antioxidant activity was also strong ($r > 0.8$). It is interesting to note that in our previous study [9,10], the extracts with the highest concentration of phenolics and flavonoids demonstrated low or even lacking antimicrobial activity.

For the plant extracts, on the other hand, there was weak correlation between phenolic content and antiradical activity ($r = 0.19$ for *S. scardica*; $r = 0.24$ for *P. major*) and between phenolic content and FRAP value ($r = 0.38$ for *S. scardica*;

Table 2

Amount of extracted phenolics and flavonoids, radical scavenging activity and ferric reducing power of plant extracts

Solvent	*S. scardica* Total flavonoids [mg RE/g]	*S. scardica* Total phenolics [mg CAE/g]	% RSA	FRAP value [µmol Fe^{2+}/g dry weight]	*P. major* Total flavonoids [mg RE/g]	*P. major* Total phenolics [mg CAE/g]	% RSA	FRAP value [µmol Fe^{2+}/g dry weight]
70% EtOH	12.30 ± 0.90	54.20 ± 1.61	59.1 ± 2.9	364.7 ± 4.6	11.33 ± 0.75	55.53 ± 0.25	64.6 ± 3.3	366.2 ± 6.6
XX:Gly 1:2	10.20 ± 0.40	45.67 ± 2.69	22.3 ± 1.4	158.9 ± 14.9	3.60 ± 0.30	25.00 ± 1.13	28.5 ± 0.4	173.1 ± 3.1
CiA:PrD 1:4	8.70 ± 0.24	41.60 ± 0.75	32.8 ± 2.1	171.4 ± 0.7	6.90 ± 0.42	30.50 ± 0.28	33.5 ± 1.4	200.8 ± 7.8
XX:Ur 1:1	11.85 ± 0.45	56.10 ± 1.80	28.6 ± 1.6	182.8 ± 5.5	8.85 ± 1.05	46.35 ± 1.05	35.1 ± 2.2	172.8 ± 3.3
XX:Glu:H_2O 5:2:25	12.00 ± 0.65	62.70 ± 1.50	46.1 ± 2.1	274.0 ± 9.1	6.60 ± 0.49	69.90 ± 3.60	67.3 ± 2.8	326.6 ± 8.0
XX:PrD 1:3	8.60 ± 0.14	57.45 ± 0.45	46.4 ± 2.5	248.7 ± 4.8	3.60 ± 0.24	57.00 ± 1.20	47.2 ± 0.9	270.0 ± 1.7
Bet:MA:H_2O 1:1:6	10.00 ± 0.37	59.70 ± 0.88	35.4 ± 2.3	217.4 ± 7.8	7.80 ± 0.00	65.30 ± 0.37	45.0 ± 2.2	307.5 ± 16.3
XX:Xy 4:1	4.68 ± 0.35	25.00 ± 1.32	44.2 ± 1.2	222.4 ± 3.6	4.08 ± 0.15	22.19 ± 0.15	67.2 ± 2.1	299.2 ± 14.3
LA:PrD 1:1	8.24 ± 0.50	33.68 ± 0.19	37.7 ± 1.9	285.0 ± 11.8	4.38 ± 0.00	32.26 ± 1.73	50.3 ± 2.2	349.3 ± 9.0
XX:PrD:H_2O 1:1:1	4.49 ± 0.20	17.73 ± 0.56	32.1 ± 2.3	169.3 ± 3.7	2.30 ± 0.08	25.08 ± 1.28	39.6 ± 1.7	227.7 ± 6.9
LA:Fr 5:1	4.08 ± 0.00	31.80 ± 1.02	41.8 ± 1.8	282.6 ± 10.4	6.34 ± 0.31	34.34 ± 0.35	42.8 ± 1.1	259.8 ± 10.1

Table 3

Amount of extracted phenolics and flavonoids, radical scavenging activity and ferric reducing power of propolis extracts

Solvent	Total flavonoids (% of raw propolis)	Total phenolics (% of raw propolis)	% RSA	FRAP value [μmol Fe^{2+}/g dry weight]
70% EtOH	11.3 ± 0.3	37.1 ± 1.2	48.1 ± 1.4	14906 ± 187
CiA:PrD 1:4	9.7 ± 0.1	36.7 ± 0.7	36.0 ± 0.2	12389 ± 318
XX:Glu:H2O 5:2:25	4.9 ± 0.4	14.7 ± 0.3	20.3 ± 0.6	4646 ± 26
Bet:MA:H2O 1:1:6	5.5 ± 0.3	20.1 ± 2.1	26.8 ± 0.7	5855 ± 142
XX:Ur 1:1	8.2 ± 0.3	26.5 ± 0.5	34.4 ± 1.3	5456 ± 112
LA:PrD 1:1	11.0 ± 0.1	38.3 ± 0.5	36.4 ± 0.3	13236 ± 501
XX:Gly 1:2	8.4 ± 0.0	26.3 ± 0.3	43.5 ± 0.5	8783 ± 193
XX:PrD:H2O 1:1:1	11.4 ± 0.4	39.8 ± 0.7	47.4 ± 0.7	12259 ± 363
XX:Xy 4:1	7.5 ± 0.3	23.9 ± 0.7	40.0 ± 0.8	9776 ± 118
XX:PrD 1:3	10.7 ± 0.1	37.0 ± 0.7	50.3 ± 0.2	12321 ± 321
La:Fr 5:1	8.6 ± 0.2	30.8 ± 0.5	34.6 ± 1.0	5933 ± 208

$r = 0.44$ for *P. major*). The correlation was weaker in the case of mountain tea *S. scardica*. Weak correlation was observed also between flavonoid content and antioxidant activity (for DPPH and FRAP $r < 0.2$). As already mentioned, the chemical features of the phenolics and flavonoids in both plants and propolis are different, and this could be the reason for these distinct antioxidant characteristics. Interestingly, some authors have observed a strong negative correlation between antioxidant activity and TPC of NADES extracts [17]. Thus, specific interactions between extracted compounds and the ingredients of the NADES could also be of importance with respect to antioxidant activity.

Conclusions. The results obtained confirm that NADES extracts of medicinal plants and propolis have a good antioxidants potential, which allows obtaining natural antioxidants without the application of organic solvents. Because of the biocompatibility and low toxicity of the NADES, the opportunity to apply these extracts directly in formulations, such as cosmetics, food supplements, etc., seems promising. Obviously, further in-depth studies are necessary in order to clarify the influence of the NADES on the antioxidant activity of the dissolved substances and to confirm that such NADES extracts could be used in pharmaceutical and food industry.

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Institute of Organic Chemistry
with Centre of Phytochemistry
Bulgarian Academy of Sciences
Akad. G. Bonchev St, Bl. 9
1113 Sofia, Bulgaria
e-mails: Hristo.Petkov@orgchm.bas.bg
Boryana.Trusheva@orgchm.bas.bg
Slaveya.Krustanova@orgchm.bas.bg
Tsvetinka.Grozdanova@orgchm.bas.bg
Milena.Popova@orgchm.bas.bg
Kalina.Alipieva@orgchm.bas.bg
Vassya.Bankova@orgchm.bas.bg