FACTA UNIVERSITATIS
Series: **Physics, Chemistry and Technology** Vol. 18, Nº 2, 2020, pp. 89 - 97
https://doi.org/10.2298/FUPCT2002089M

SAMBUCUS RACEMOSA L. FRUIT EXTRACTS OBTAINED WITH CONVENTIONAL AND DEEP EUTECTIC SOLVENTS[†]

UDC 577.13 : 581.192.2

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Abstract. *This study aimed to provide information on the extraction yields, total phenols, total flavonoids, antioxidant activity, and qualitative-quantitative chemical composition of the extracts obtained from the dried fruit of Sambucus racemosa L. with conventional solvents (methanol, acetone, chloroform) and deep eutectic solvents (choline chloride urea and choline chloride glycerol). Solid-liquid extraction, Folin-Ciocalteu method, DPPH free radical scavenging, HPLC-UV/VIS were the methods used in this study. The highest extraction yield (18%) was obtained with ChCl:G as an extragent. The highest amount of total flavonoids was the content of this extract (12.3 mg QE/g d.e.). Extracts obtained with acetone and ChCl:U possess the highest amount of total phenol content (125.26 and 104.38 mg GAE/g d.e. respectively). All tested extracts showed free radical scavenging activity. Identified components of extracts are gallic acid, rutin, caftaric acid, caffeic acid, hyperoside, vitexin, and quercetin. Fruit extracts of S. racemosa are a rich source of phenols and flavonoids. The extracts obtained with DESs are more active in free radical scavenging than those obtained with conventional solvents, and possess qualitatively and quantitatively more components, apart from vitexin that was detected only in the methanol extract.*

Key words: *red elderberry, extraction, choline chloride urea, choline chloride glycerol*

Received December 18th, 2020; accepted December 27th, 2020
[†] Acknowledgement: The research leading to these results has received funding from the Republic of Serbia - Ministry of Education, Science and Technological Development, Program for Financing Scientific Research Work, no. 451-03-68 / 2020-14 / 200133 and 174007.
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1. INTRODUCTION

The red elderberry (*Sambucus racemosa* L.) is classified to the Caprifoliaceae family (Gleason and Cronquist, 1991). It is a large deciduous shrub or small tree which have small bright red, fleshy berries grouped in large clusters that appear in summer. It is known that they have been used on the southern Northwest Coast of North America through much of the Holocene (Losey et al., 2003). They colonize most areas along railways, roadways, forest edges, and fruits are highly desirable food to birds (Stang, 1990; USDA, NRCS 2019). An interesting fact is that the red elderberry (*S. racemosa*) is the most common and reliable shrub indicator of O_3 phytotoxic effects (Manning, 2005).

The natural sources of antioxidant compounds present a highly valuable material in pharmaceutical industries. Elderberry is one of the richest in anthocyanins, polyphenolics, and flavonols glycosides, and all of these chemical compounds contribute to the high antioxidant capacity (Thole et al., 2006). The fruits of red elderberry are good sources of phytochemicals, such as phenolic compounds (Mikulic-Petkovsek et al., 2014). These compounds have antioxidant properties, and recent data indicate that they help in optimizing human health by neutralizing free radicals (Radovanović et al., 2018).

Seeking new solvents, which would be an adequate substitute for common organic solvents that are characterized as toxic and highly volatile, is in progress. In the past two decades, ionic liquids (ILs) have gained much interest from the scientific community (Rogers & Seddon, 2003). Deep eutectic solvents (DES) are considered as an alternative to ILs. In the characteristics, DESs are similar to ILs, but cheaper to produce (lower cost of the raw materials), less toxic, and often biodegradable (Carriazo et al., 2012). It is considered that these types of liquids are ideal substitutes for conventional organic solvents (Dai et al., 2013a; Troter et al., 2018). By the definition, they may also have an ionic character but consist of a mixture of organic compounds having a melting point significantly lower than that of either individual component (Clouthier and Pelletier, 2012). Most commonly used DESs are based on choline chloride (ChCl), carboxylic acids, and other hydrogen-bond donors, e.g., urea, succinic acid, citric acid, and glycerol. It has been reported that between glycerol and ChCl in a molar ratio 2:1 DES can be formed (Gorke et al., 2008), and also in the same molar ratio between urea and ChCl (Gutierrez et al., 2010).

To the best of our knowledge, no comparative studies on phenolic composition, antioxidant activity, and chemical composition of extracts of *S. racemosa* fruit extracted with conventional and DESs have been performed before.

2. MATERIALS AND METHODS

2.1. Plant material

The berries of *S. racemosa* were collected on mountain slopes of Stara Planina at an altitude of 1600-1700 m above sea level at their optimum fruit maturity during August 2018. The samples of fruits were put into plastic bags, which were labeled, and stored in a freezer at -20 °C until further analysis. Voucher No. 13899 was deposited in the Herbarium at the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Republic of Serbia. Marija S. Markovic, Ph.D. identified the plant material. Dried berries were ground in a Braun® cutting mill and then separated from seeds by sifting through the sieve (0.2-0.4 mm).

2.2. Chemicals

The following standards were used: gallic acid, rutin, caftaric acid, caffeic acid, hyperoside, vitexin, and quercetin from Sigma-Aldrich Chemie GmbH. Choline chloride (Sigma Aldrich, ≥ 98%), urea (Zorka, Šabac, Serbia, 99.5%), glycerol (MeiLab Belgrade, Serbia, Ph Eur grade), methanol (Zorka-Pharma, Šabac, Serbia, 99.5%), absolute ethanol (Sigma Aldrich, St. Louis, USA, 99.5%), chemicals for the mobile phases were HPLC grade acetonitrile and formic acid (Fluka Chemie GmBH), water for the mobile phase was double distilled and purified with the Milli-Q system (Millipore, Bedford, MA).

2.3. Preparation of the extracts

Dried and ground fruits of red elderberry (1 g) were extracted with 10 cm^3 of the corresponding solvent (acetone, methanol, chloroform, choline chloride glycerol (ChCl:G), or choline chloride urea (ChCl:U)). DESs were prepared as reported before (Đorđević et al., 2018). It is known that viscosity hinders the efficiency of DES as extraction solvents because it results in a slow mass transfer. This problem was solved as the extraction conditions (temperature) were adjusted to reduce the viscosity of DES and improve the yield (Dai et al., 2013b). The extraction was carried out 180 min at 60 °C in a thermostated chamber with constant stirring. After the extraction was done, the fruit extracts were centrifuged for 10 min at 10.000 rpm, and each supernatant was filtered through a Chromafil AO-20/25 polyamide filter manufactured by Macherey-Nagel (Düren, Germany). The extractions were done in triplicate.

2.4. Extraction yields, total phenols, and total flavonoids

Extraction yields were expressed as a percentage of milligrams of extract per gram (dry weight) of fruits. The total phenolics contents were determined using the Folin-Ciocalteu method (Brighente et al., 2007), with some modifications. By this method, the reaction mixture was composed of 0.1 cm^3 of extract, 5.4 cm^3 of distilled water, and 0.5 cm^3 of the Folin-Ciocalteu reagent. After 3 min, 1.0 cm^3 of a saturated sodium carbonate solution was added. These mixtures were shaken and allowed to stand for 1 h at room temperature. The absorbance against the reagent blank was measured at λ = 725 nm (each measurement was repeated three-times) with a UV-Visible spectrophotometer (JENWAY 6305, UK). Based on the absorbance of standard solutions of gallic acid, concentrations (mg/ml) of phenolic compounds were determined using the equation $y = 0.834x + 0.004$ ($r^2 = 0.982$). Total phenols were estimated as gallic acid equivalents (GAE) and expressed as mg gallic acid equivalents/1 g (dry weight) of the extract (Singleton et al., 1999).

For the spectrophotometric determination of total flavonoid content, a modified method, according to the already reported method (Brighente et al., 2007), was used. Namely, 1.5 cm^3 of 2% aluminum chloride ($AlCl_3$) ethanol was mixed with the same volume of the extract. The absorbances were measured at λ = 415 nm after 1 h against a blank (ethanol). Based on the absorbance of standard quercetin solution, concentrations (mg/ml) of flavonoids compounds were determined using the equation $y = 39.90x - 0.007$ ($r^2 = 0.999$). Total flavonoids were estimated as quercetin equivalents (QE), expressed as mg quercetin equivalents/ 1 g (dry weight) of extract.

2.5. Determination of the antioxidant activity with the DPPH free radical scavenging method

The percentage of antioxidant activity of extracts was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical assay. First, the solutions of the extracts in ethanol were prepared. The ethanol solution of the DPPH radical (1 cm^3, 3×10^{-4} mol/dm^3) was added to the ethanol solutions of the extracts (2.5 cm^3). This mixture was incubated at room temperature for 20 min. The changes in color (from deep violet to light yellow) were read at λ =517 nm using a UV/VIS spectrophotometer (JENWAY 6305, UK). The absorbance was also read for the ethanol solution of the DPPH radical (1 cm^3 of DPPH radical at the determined concentration with 2.5 cm^3 of ethanol), as well as for the ethanol solution of each extract without the DPPH radical (2.5 cm^3 of the extract with 1 cm^3 of ethanol). Pure ethanol presented a blank solution. The scavenging activity of the free radicals percentage (*AA*%) was determined according to the formula:

$$AA(\%) = 100 - [(A_U - A_B * \frac{100}{A_K})] \quad (1)$$

where A_U is the absorbance of the ethanol solution of the extract treated with DPPH radical, A_B is the absorbance of an ethanol solution of the extract not treated with DPPH radical and A_K is the absorbance of ethanol solution of the DPPH radical (diluted as 1 cm^3 DPPH radical (concentration of 3×10^{-4} mol/dm^3) with 2.5 cm^3 of ethanol).

All tests were run in duplicate, and for each concentration, the average absorption was noted. The IC_{50} value, which is the concentration of the test material that inhibits 50% of the free radical concentration, was calculated as mg/ml (Kumarasamy et al., 2007; Stanojević et al., 2017). With an absorption maximum at λ = 517 nm, DPPH radical is a stable radical. An antioxidant via electron or hydrogen atom transfer reactions can reduce DPPH radical to the hydrazine derivative, which results in a decrease of absorption maximum (Lu and Foo, 2001). Defined as the concentration required to scavenge 50% of the available free radicals, IC_{50} values were estimated by nonlinear regression for all the extracts.

2.6. HPLC analysis

The extracts were analyzed by an Agilent Technologies 1100 Series chromatograph equipped with a degasser, a binary pump, a thermostated column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm), and a UV/VIS detector. The samples were dissolved in methanol. All solvents and samples were filtered by a 0.45 μm Millipore filter. The volume of the injected sample was 5 μl. The mobile phase was made from (A) 0.1% formic acid in water and (B) acetonitrile. The next linear gradient program at a flow rate of 0.5 ml/min has been applied: 20% (B) at the start, then 90% (B) for 40 min, and five more minutes with 90% (B). The column temperature was 25 °C.

2.7. Statistical analysis

The data are presented as mean value ± standard deviation (SD). Statistical analysis was performed using Student's t-test for nondependent samples. The statistical analyses were calculated using SPSS 10.0.1. (SPSS Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

3.1. Extraction yields, total phenols, and total flavonoids

The results of extraction yields, total phenols, and total flavonoids are presented in Table 1. According to them, the highest extraction yield was obtained with ChCl:G as extragent. Also, extract obtained with ChCl:G as extragent contained the highest amount of total flavonoids. Extracts obtained with acetone and ChCl:U possess the highest amount of total phenol content.

Extraction yields obtained in this study with conventional solvents and ChCl:U are lower than those obtained by Duymus et al. (2014), who used conventional solvents for the extraction of the fruit of *S. racemosa*. It is most probably the result of a longer extraction time (120 h in comparison with 3 h), although, reported by Salamon et al. (2015), the long extraction time is not crucial for extraction of *S. racemosa* fruit. The extraction with ChCl:G provided the extraction yield of 18%, which is higher than the value of 16.1% achieved for a 40-fold longer period when water is used as a solvent for the extraction (Duymus et al., 2014).

The extraction of flavonoids is related to the polarity of the solvent used for the extraction. The less polar solvents (such as chloroform) are useful to extract aglycone flavonoids, and more polar solvents (acetone, ethyl acetate, alcohol, and water) are used to extract glycoside flavonoids (Mabry et al., 1970).

Table 1 Extraction yield, total phenols and total flavonoids in fruit extracts of *Sambucus racemosa* L. obtained with different solvents

A solvent used for the extraction	Extraction yield (%)	Total phenols (mg GAE/g d.e.)	Total flavonoids (mg QE/g d.e.)
Methanol	15.2 ± 0.2	84 ± 1	8.5 ± 0.1
Acetone	6.5 ± 0.2	125 ± 2	11.0 ± 0.3
Chloroform	9.6 ± 0.07	41.8 ± 0.8	7.5 ± 0.2
ChCl:U (1:2)	6.0 ± 0.70	104 ± 1	8.5 ± 0.2
ChCl:G (1:2)	18.0 ± 0.04	62.6 ± 0.4	12.0 ± 0.3

The lowest amount of the total phenols and total flavonoids was, as expected, in the chloroform extract, because chloroform is known as a not very efficient solvent for their extraction. Chebil et al. (2007) determined the solubility of quercetin in different conventional solvents, including acetone. They found that solubility of quercetin is the highest in acetone, of all tested solvents, and our results of total flavonoids expressed as QE show a higher amount of flavonoids in ChCl:G than in the acetone extract.

Because DESs are solvents for both polar and nonpolar metabolites (Biswas et al., 2006), it implies that they can be applied as a solvent for the extraction of many types of natural compounds, such as phenolic compounds (Paiva et al., 2014). Results of Paiva et al., who were extracting phenolic compounds from green coffee beans with acetone and different DESs based on choline chloride, show that total phenolic content in extract varies with different DES. Our results agree with theirs, because DESs with the same cation was used in this study, and acetone provided more total phenols in extract then DESs used. The difference in total phenols was noticed. The observed difference in the total phenols in the extract is correlated to the anion of DESs, and the efficiency of

phenolic compound extraction (such as gallic acid, quercetin (Du et al., 2009) and rutin (Zeng et al., 2010) is anion-dependent (Dai et al., 2013b).

3.2. DPPH radical scavenging activity

All extracts tested in this experiment showed free radical scavenging activity. Based on the results obtained in this study (Fig. 1) the ChCl:U extract was shown as the most active free radical scavenger (IC_{50} value 58 μg/cm^3), followed by ChCl:G extract (IC_{50} value 138 μg/cm^3). The extracts obtained with conventional solvents (methanol, acetone, chloroform) had lower radical scavenging activity, which means higher IC_{50} values: 217 μg/cm^3, 1391 μg/cm^3, 2940 μg/cm^3, respectively. Comparing these results with results of Duymus et al. (Duymus et al., 2014) who provide results of antioxidant activities of extracts of *S. nigra* extracted with water, 70% ethanol, 70% acetone, and methanol, it is evident that ChCl:U provides an extract of *S. racemosa* with better antioxidant activity. The methanol extract of *S. racemosa* fruit showed in this study lower antioxidant activity than methanol extract of *S. nigra* fruit obtained by other authors. The other research group used DES as an extraction solvent, ChCl:G, which provided extract with IC_{50} value equals to 138 μg/cm^3, which is comparable with methanol and water extract which had IC_{50} values 117 μg/cm^3 and 123 μg/cm^3, respectively (Duymus et al., 2014). It should beared in mind that these authors spent 40-fold more time on the extraction process.

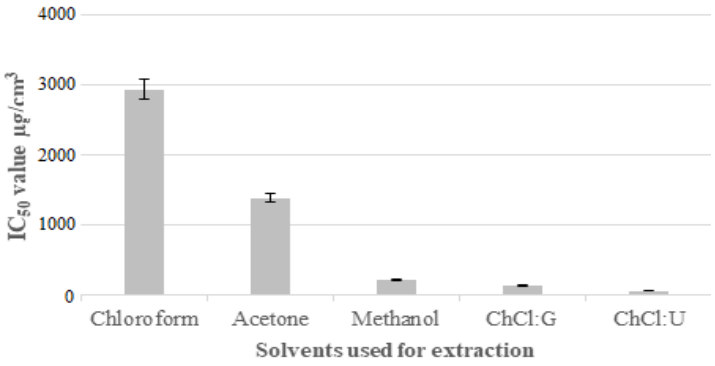

Fig. 1 IC_{50} values of extracts of *S. racemosa* fruit extracts obtained with different solvents

The reason for better antioxidant activities of the extracts obtained with DESs compared to the extracts obtained with conventional solvents could also be the ability, which DESs have, of donating and accepting protons and electrons, which results in the forming of hydrogen bonds, and therefore, increasing their dissolution capability (Zhang et al., 2012).

Flavonoids are believed to be responsible for antioxidant activity. The highest content of total flavonoids was in the ChCl:G extract, followed by the acetone extract. However, any direct correlation between these two properties was not demonstrated. Comparing the content of flavonoids and antioxidant activity of different extracts seems reasonable because while it is possible to find out the direct correlation between the concentration of pure compounds and the antioxidant capability of their solutions, it is not easy when plant extracts is used (Rakotoarison et al., 1997; Moure et al., 2001). The chemical content of

the samples does not always correlate with their antioxidant power of extracts is the statement accepted in general by the authors (Barros et al., 2011).

3.3. HPLC analysis

The qualitative-quantitative analyses, carried out using an HPLC apparatus coupled with a UV detector, of the extracts tested in this study gave the outcomes presented in Table 2. Identification was made by comparison of the retention time and UV spectra of authentic standards, and quantitative data were calculated from calibration curves of the standard solutions.

Gallic acid was detected and quantified in all extracts of *S. racemosa* fruits. The ChCl:G and ChCl:U extracts of *S. racemosa* were found to be the richest in gallic acid, with concentrations 9.18 and 5.96 mg/g (extract dry weight), respectively. With these concentrations, gallic acid is the compound with the highest content in the extracts. The calculated values are several times higher than the values for the extracts obtained with conventional solvents. For comparison, the concentration of gallic acid in methanol extract was 0.71 mg/g (dry weight) of the extract.

The highest concentration of rutin in *S. racemosa* extract was observed by ChCl:U, followed by methanol and ChCl:G. This polyphenol was also identified by Mikulic-Petkovsek et al. in *S. racemosa* extract (Mikulic-Petkovsek et al., 2015).

Table 2 Identified compounds and their concentrations in the extracts of *S. racemosa* L. fruits obtained with different solvents

Compound number ($t_{ret.}$/min)	Name	Confirmation	UV/Vis (nm)	Metanol (mg/g d.e.)	Chloroform (mg/g d.e.)	ChCl:U (mg/g d.e.)	ChCl:G (mg/g d.e.)
1. (8.90)	Gallic acid	Standard	350.4	0.71	1.08	5.96	9.18
2. (11.85)	Rutin	Standard	350.4	0.52	-	1.17	0.40
3. (12.76)	Caftaric acid	Standard	350.4	-	-	1.03	0.37
4. (13.39)	Caffeic acid	Standard	350.4	0.58	-	2.04	0.67
5. (14.12)	Hyperoside	Standard	350.4	-	-	1.49	0.50
6. (14.37)	Vitexin	Standard	350.4	0.83	-	-	-
7. (19.82)	Quercetin	Standard	350.4	-	-	1.64	0.56

Abbreviations for solvents: ChCl:U, choline chloride urea; ChCl:G, choline chloride glycerol; -, not detected.

Caftaric acid was a constituent of *S. racemosa* fruit extracts only in the cases when DESs were used as the solvent. Namely, ChCl:U and ChCl:G provided extracts with caftaric acid in concentrations 1.03 and 0.37 mg/g (dry weight) of extract, respectively. A derivative of hydroxycinnamic acid, caffeic acid, was identified in both DESs and methanol extracts. The highest concentration of caffeic acid was found to be in the extract of *S. racemosa* fruits obtained with ChCl:U, followed with ChCl:G and methanol. The presence of caffeic acid in elderberry extracts was expected (Mikulic-Petkovsek et al., 2015). Hyperoside was not detected in *S. racemosa* fruit extracts obtained by conventional solvents, which is in agreement with results of Mikulic-Petkovsek et al. (Mikulic-Petkovsek et al., 2015). However, the extracts obtained with ChCl:U and ChCl:G had concentrations of hyperoside 1.49 and 0.5 mg/g dry weight of the extract, respectively. Vitexin was present only in the methanol extract of *S. racemosa* fruits with a concentration 0.83 mg/g dry weight of the extract. When it comes to quercetin, unlike Mikulic-Petkovsek et al. (2015), who identified it in the extracts of *S.*

racemosa fruit which were prepared with conventional solvents, the herein investigated extracts contained this compound only when DESs were used. ChCl:U extract had 1.64 and ChCl:G extract had 0.56 mg/g dry weight of extract, respectively. An evident interaction between quercetin and DESs has been demonstrated by Dai et al. (Dai et al., 2013a).

4. CONCLUSION

Red elderberry as a source of bioactive agents is not investigated sufficiently, so the analysis of the qualitative and quantitative composition of *S. racemosa* fruit extracts and their free radical scavenging activity was performed. The results were compared, and conclusions are drawn. Fruit extracts of *S. racemosa* are a rich source of phenols and flavonoids. The DESs provided extracts that are more active in free radical scavenging than those obtained with conventional solvents. To the best of our knowledge, there are no results for the free radical scavenging activity of *S. racemosa* fruit extracts from other authors. Different active phenolic compounds, such as gallic acid, rutin, caftaric acid, caffeic acid, hyperoside, vitexin, quercetin are components of the extracts. The concentrations of these compounds were determined. The DESs used in this study (ChCl:U and ChCl:G) extracted more different compounds than conventional solvents did. These compounds were present in DESs extracts in higher concentrations than in the conventional solvent extracts, with an exception of rutin, which was present in higher amount in the methanol extract than in ChCl:G. Vitexin was only extracted with methanol. Our results demonstrate that DESs are both greener and more efficient substitution to conventional solvents for the extraction of bioactive compounds from *S. racemosa* fruits.

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EKSTRAKTI PLODA CRVENE ZOVE DOBIJENI PRIMENOM KONVENCIONALNIH I EUTEKTIČKIH RASTVARAČA

Cilj ovog rada je da prikaže rezultate koji se tiču prinosa ekstrakcije, sadržaja ukupnih fenola, ukupnih flavonoida, antioksidativne aktivnosti i kvalitativno-kvantitativnog hemijskog sastava ekstrakata osušenog ploda crvene zove (Sambucus racemosa L.) dobijenih primenom konvencionalnih rastvarača (metanol, aceton, hloroform) i eutektičkih rastvarača (holin-hlorid/urea i holin-hlorid/glicerol). Čvrsto-tečna ekstrakcija, Folin-Ciocalteu metoda, DPPH metoda, HPLC-UV/VIS su metode koje su korišćene u radu. Najveći prinos ekstrakcije (18%) postignut je primenom ChCl:G kao ekstragensa. Taj ekstrakt je imao najveći sadržaj ukupnih flavonoida (12,3 mg QE/g suvi ekstrakt). Ekstrakti dobijeni acetonom i ChCl:U su imali najveći sadržaj ukupnih fenola (125,26 i 104,38 mg GAE/g suvi ekstrakt). Svi ekstrakti su pokazali antioksidativnu aktivnost. Identifikovani sastojci u ekstraktima su galna kiselina, rutin, kaftarinska kiselina, kafena kiselina, hiperozid, viteksin i kvercetin. Ekstrakti ploda crvene zove su bogat izvor fenola i flavonoida. Ekstrakti dobijeni eutektičkim rastvaračima su pokazali veću antioksidativnu aktivnost od onih dobijenih konvencionalnim rastvaračima, i poseduju i u kvalitativnom i kvantitativnom smislu više sastojaka, osim viteksina koji je bio prisutan samo u metanolnom ekstraktu.

Ključne reči: *crvena zova, ekstrakcija, holin-hlorid/urea, holin-hlorid/glicerol*