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Effect of solvents on phenolic compounds extraction and antioxidant activity of *Prunus spinosa* L. fruits

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Abstract: The aim of this work was quantification of phenolic compounds and determination of antioxidant activity of *Prunusspinosa* fruit extracts. Extractions of phenolic compounds were carried out with water and four alcohol mixture (50% methanol, 50% ethanol, 80% methanol, and 80% ethanol). Spectrophotometric determination of phenolic compounds was done by Folin-Ciocalteu method and flavonoids with $AlCl_3$ method. Arnow reagent was used in determination of phenolic acid content while anthocyanin content was determined with pH differential method. Butanol-HCl assay was applied to determine proanthocyanidin content. Investigated phenolic compounds were in the range of 14.02-30.20 mg GAE/g dw (total phenolic compounds), 0.789-1.538 mf RE/g dw and 0.450-1.039 mg QE/g dw (flavonoids), 4.55-7.24 mg CAE/g dw (phenolic acids), 0.361-1.05 mg CGE/g dw (anthocyanins), 3.97-26.49 mg CE/g dw (proanthocyanins). The highest content of investigated compounds was found for 50% ethanol extract (except anthocyanins) and the lowest content was in water extract. The highest antioxidant activity had 50% ethanol extract for all antioxidant methods. Very high correlations were found between antioxidant activity and content of all analyzed compounds.

INTRODUCTION

Blackthorn (*Prunus spinosa* L.) is a deciduous scrub or small tree, widespread in Europe, western Asia and Northwest Africa. Fruits are known for their astringent properties. It is also used in treatment of different health conditions such as cough, diarrhea, constipation, and inflammation. Fruit possess antiseptic properties and have relaxing effects on stomach inflammation (Lust, 1980; List and Horhammer, 1971; Tardio, Pardo de Santayana, Morales, 2006). In addition to its medicinal use, fruits are also used in the food industry for the production of jams, wine, tea or juices (Veličković, Stojanović, Kostić *et al*., 2014).

In recent years wild fruits were intensively investigated due to their antioxidant properties and increasing demand for finding new natural antioxidants for food industry and pharmaceutical production (Carocho and Ferreira, 2013; Egea, Sancez-Bel, Romojaro, *et al.*, 2010). Blackthorn has been recognized by several research groups as a new source of antioxidants. It has been reported that fruits are rich source of phenols, including phenolic acids, anthocyanins and flavanols (Guimarães, Barros, Dueñas, *et al.*, 2013; Barros, Carvalho, Morais, *et al.*, 2010; Jabłońska-Ryś, Zalewska-Korona, Kalbarczyk, 2009) It was pointed out that these compounds play a significant role in the antioxidant capacity of the plant extracts (Ruiz-Rodriguez, de Ancos, Sancez-Moreno, *et al.*, 2014; Ganhão, Estévez, Kylli *et al.*, 2010; Egea *et al.*, 2010). The aim of this study was quantitative determination of phenolic compounds in fruit extracts. Antioxidant activity of the extracts was tested by DPPH, ABTS and FRAP method using Trolox as a standard. Extracts obtained with different solvent systems (water, 50% methanol, 50% ethanol, 80% methanol, 80% ethanol) were investigated. To the best of our knowledge, this is the first report on

phenolic content and antioxidant activity of *P. spinosa* fruit extracts prepared with different solvents from selected region. Obtained data make an important contribution to the understanding of the chemical potential of this valuable species for industrial application.

EXPERIMENTAL

Chemicals used in this work were of analytical grade and were purchased from Sigma-Aldrich Chemical Company (Germany) except potassium chloride and ferrous ammonium sulfate (Kemika Zagreb, Croatia) and butanol (Merck Chemical Suppliers, Germany).

Plant material

Fruit sample of *Prunus spinosa* L. was collected in the area of Maglaj region (Bosnia and Hezegovina) at the end of October 2016. Identification of the species was done by Prof. Dr. Nedad Bašić, a plant taxonomist. Voucher specimen was deposited at Department of Ecology, Faculty of Forestry University of Sarajevo. The pulp was separated from the seeds and was dried in the oven at 40°C and stored in a paper bag in the herbarium. Before analysis, the sample was powdered in an electric mill.

Extraction

Fruit extracts were prepared with solvents of different polarity by ultrasound extraction. (Ultrasound bath, Elmecs, Italy). Extractions were done with five solvents/mixture: distilled water, 50% aqueous methanol, 50% aqueous ethanol, 80% aqueous methanol, and 80% aqueous ethanol. The fruit sample (0.5 g) was extracted with an appropriate solvent (12 mL) for 30 minutes at room temperature. Obtained extract was centrifuged at 3000 rpm for 10 minutes and supernatant was separated from solid material. The residue was extracted once more with the same aliquot of solvent. Obtained supernatants were combined and volume was brought to 25 mL for each extract in a volumetric flask. Prepared extracts were kept at -20°C until analysis.

Determination of total phenolic compounds

Folin-Ciocalteu method described by Singelton, Orthofer, Lamuela-Raventos (1974) with Folin-Ciocalteu reagent, and gallic acid as a standard, was used to determine total phenolic compounds (TF). Reaction mixture was allowed to stand for 30 minutes in a water bath at 40°C prior measurements. Absorbance of standards and samples was measured at 765 nm. Results are expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g dw). All spectrophotometric measurements were done with Shimadzu UV-mini 1240 spectrophotometer.

Determination of total flavonoids

Total flavonoids (TF) were determined by aluminium chloride method described by Quettier, Gressier, Vasseur, *etal.* (2000) and Ordonez, Gomez, Vattutuone, *et al.* (2006). Rutin and quercetin were used as standards. Samples were incubated at room temperature for an hour and absorbance was measured at 415 nm (rutin) and at 420 nm (quercetin). Absorbance was corrected with a sample blank (sample prepared without addition of the reagent) Results are expressed as mg rutin or quercetin equivalents per gram of dry weight (mg RE/g dw and mg QE/g dw).

Determination of total phenolic acids

Total phenolic acids (TPHA) were determined using Arnow reagent and the method given by Gawlic-Dziki (2012). Caffeic acid was used as a standard and absorbance was measured at 490 nm. Blank sample without addition of the reagent was used for correction of the absorbance. The results were expressed as mg caffeic acid equivalents per gram of dry weight (mg CAE/g dw).

Determination of total proanthocyanidins

Total proanthocyanidins (TPA) were determined by the butanol/HCl method described by Hagerman, Harvay-Mueller, Makkar (2002). Absorbance of the sample was read at 550 nm before and after heating at 95°C for 40 minutes. Butanol/HCl mixture was used as a blank. The results were expressed as mg of cyanidin chloride equivalents per gram of dry weight (mg CE/ g dw).

Determination of monomeric anthocyanins

Determination of total monomeric anthocyanins (TMA) was carried out by pH differential method described by Lee, Durst, Wrolstad (2005). Absorbance was measured at 520 nm and 700 nm and molar extinction coefficient of cyanidin-3-O-glucoside (26900 L/mol·cm) and molar weight (MW) (449.2 g/mol) were used for calculations. Total monomeric anthocyanins (TMA) were expressed as mg of cyanidin-3-glucoside equivalents per gram of dry weight (mg CGE/ g dw).

Determination of antioxidant activity

DPPH assay

Antioxidant activity with $DPPH^{\bullet}$ radical (1,1-diphenyl-2-picrylhydrazyl radical) was measured by the method of Brand-Williams, Cuvelier, Berset (1995), and Thaipong, Boonprakob, Crosby, *et al.* (2006). The extracts, DPPH stock solution and Trolox standards were prepared in methanol. DPPH working solution was prepared on a daily baseand diluted to absorbance of 1.10±0.02 at 515 nm. Decrease in absorbance of reaction mixture was measured after 30 minutes. Final results were expressed as µmol of Trolox equivalents per gram of dry weight (µmol TE/g dw).

ABTS assay

The method of Ree, Pellegrini, Proteggente, *et al.* (1999) modified by Thaipong, *et al.* (2006) was used in order to perform ABTS assay. $ABTS^{\bullet+}$ (2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt) solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate solution, 12-16 hours prior to analysis. $ABTS^{\bullet+}$ solution was further diluted with methanol till absorbance of 1.10 ±0.02 units was reached at 734 nm. Reduction in absorbance of reaction mixture was monitored after 6 minutes. As a standard, methanolic solutions of Trolox were used. Results were expressed as µmol of Trolox per gram of dry weight (µmol TE/g dw).

FRAP assay

Ferric reducing antioxidant power (FRAP) was determined by method of Benzie and Strain (1999). FRAP reagent is a mixture of TPTZ (2,4,6-tripiridil-s-triazine) acidic solution, acetate buffer (pH=3.6) and $FeCl_3$ solution mixed in the ratio 10:1:1. The solution was heated at 37°C for 30 minutes in a water bath. Plant extracts or Trolox standards (0.1 mL) after addition of FRAP reagent were left in the dark for 30 minutes. Absorbance of the samples/standards was measured at 593 nm against a blank. Results were expressed as µmol of Trolox per gram of dry weight (µmol TE/g dw).

RESULTS AND DISCUSSION

In this work, water, aqueous ethanolic and methanolic solutions (50% and 80% v/v) were used to extract phenolic compounds from *P. spinosa* dried fruit sample. Obtained results are presented in Table 1.

Table 1: Total phenolic compounds (TP), total flavonoids (TF), total phenolic acids (TPA), total proanthocyanidins (TPC) in *P. spinosa* fruit

Extracts	**TP** (mgGAE/g)	**TF** (mgRE/g)	**TF** (mgQE/g)	**TPA** (mgCAE/g)	**TMA** (mg CGE/g)	**TPC** (mgCE/g)
F(W)	14.02±0.08	0.789±0.002	0.450±0.002	4.55±0.03	0.361±0.005	3.97±0.01
F(M50)	29.82±0.10	1.339±0.002	0.994±0.002	6.55±0.03	0.910±0.007	22.84±0.04
F(E50)	**30.20±0.16**	**1.538±0.009**	**1.039±0.004**	**7.24±0.08**	0.973±0.004	**26.49±0.07**
F(M80)	25.14±0.19	1.332±0.007	0.860±0.001	6.09±0.02	**1.05±0.01**	19.43±0.11
F(E80)	21.19±0.14	1.184±0.003	0.715±0.001	6.12±0.01	0.866±0.004	18.03±0.04

F(w)-water, F(M50)- 50% methanol; F(E50) -50% ethanol, F(M80) -80% methanol, F(E80) -80% ethanol, the highest values in bold

The most efficient solvent for extraction of phenolic compounds (30.20 mg GAE/g), flavonoids (1.538 mg RE/g and 1.039 mg QE/g), phenolic acids (7.24 mg CAE/g) and proanthocyanins (26.49 mg CE/g) was 50% ethanol. The highest yield of TMA (1.05 mg CGE/g) was obtained with 80% methanol.

This is in agreement with the fact that aqueous mixtures of ethanol and methanol possess moderate polarity which is suitable for extraction of different phenolic compounds (Antholovich, Prenzler, Robards *et al.*, 2000). These solvents are used for extractions of flavonoids and their glycosides, phenolic acids and catechins (Tan, Tan, and Ho, 2013).

The lowest values of the contents for investigated compounds were obtained with the water extract: total phenolic compounds (14.02 mg GAE/g), flavonoids (0.789 mg RE/g and 0.450 mg QE/g), phenolic acids (4.55 mg CAE/g), monomeric anthocyanins (0.361 mg CGE/g), proanthocyanidins (3.97 mg CE/g).This can be explained by the fact that water as a solvent will extracts sugars, organic acids or soluble proteins which can interfere in the quantification of phenols (Chirinos, Rogez, Campos *et al.*, 2007). Generally, 50% ethanol and methanol were more efficient in extraction of phenols, flavonoids, phenolic acids and proanthocyanins. With anthocyanins, only 50% ethanol was more efficient in the extraction than 80% ethanol which can be explained by higher polarity of 50% ethanol and better solubility of anthocyanins in polar medium. Similar results in respect to the extractability of phenolic compounds, anthocyanins and flavonoids in different solvent systems were reported by Veličković, *et al.*, 2014. They found that extractability of 50% ethanol is higher than those of absolute ethanol for phenolic compounds (20.94 mg GAE/g), anthocyanins (0.238 mg CGE/g) and flavonoids (1.242 mg QE/g) in *P. spinosa* fresh fruit extracts. Also, water extract had the lowest yield of investigated compounds (12.17 mg GAE/g; 0.12 mg CGE/g and 1.31 mg mg QE/g) respectively.

Phenolic compounds in *P. spinosa* fruit were investigated by different research group. It was reported that total content of total phenolic compounds in fruits was 0.546-0.86 mg GAE/g fw (Uzelac, Levaj, Bursać *et al.*, 2007), 7.96 mg/g fw (Radovanović, Milenković-Anđelković, Radovanović *et al.*, 2013), 1.27 mg GAE/g fw (Egea, *et al.*, 2010); 22.55 mg GAE/g fw (Ruiz-Rodriguez, *et al.*, 2014). Flavonoid content in fruits was generally low ranging from 0.4-1.3 mg QE/g fw (Veličković, *et al.*, 2014; Radovanović, *et al.*, 2013) and 0.437-0.656 mg QE/g fw (Uzelac, *et al.*, 2007) which is in agreement with the results in this work. According to Ruiz-Rodriguez, *et al.*, (2014), average content of phenolic acids in blackthorn fruits was 7.28 mg GAE/g fw and average content of flavonols was 1.34 mg RE/g fw. Anthocyanin content in fruits was reported for methanol and ethanol extracts in the range of 0112-0.265 mg CGE/g fw (Veličković, *et al.*, 2014) and 0.305-0.497 mg CGE/g fw (Uzelac, *et al.*, 2007). We can conclude that fruits investigated in this work are a reach source of phenolic compounds.

Antioxidant activity

Antioxidant activity was investigated by DPPH, ABTS and FRAP assay and the results are given in Table 2. The highest antioxidant activity for DPPH (140.80 µmol TE/g), ABTS (223.98 µmol TE/g) and FRAP (249.13 µmol TE/g) was found in fruit extract obtained with 50% ethanol. Extracts obtained with 80% methanol and 80% ethanol had intermedium values of 118.37 and 115.12 µmol TE/g (DPPH), 171.68 and 127.60 µmol TE/g (ABTS), 193.19 and 190.04 µmol TE/g (FRAP) respectively. The lowest antioxidant activity with values of 80.59 µmol TE/g (DPPH), 85.95 µmol TE/g (ABTS) and 107.80 µmol TE/g (FRAP) had water extract. These

results show that blackthorn fruits possess high antioxidant activity. This is also supported with results of other investigators who found high values for antioxidant activity of blackthorn fruits. Our results are generally higher than those of Ruiz-Rodriguez, *et al.*, 2014 (9.2-13.9 µmol TE/g for DPPH; 18.3-76.4 µmol TE/g for ABTS and 71.1-151.7 µmol TE/g fw) and of Egea, *et al.*, 2010 (80.50 µmol TE/g fw).

Also, Gao, Bjork, Trajkovski *et al.* (2000) pointed that high antioxidant activity is usually connected with a high phytochemical content. Generally, antioxidant activity decreased in the order: FRAP>ABTS>DPPH for all extraction systems. These differences can be attributed to different radical species generated in the assays and specific reaction media (Egea, Sanchez-Bel, Martinez-Madrid, *et al.*, 2007). According to the results obtained, antioxidant activity is related to the content of phenols and solvent type used for the extraction.

Table 2: Antioxidant activity in different extracts of *P. spinosa* fruit

	DPPH (µmolTE/g)	ABTS (µmolTE/g)	FRAP (µmolTE/g)
F(W)	80.59±0.16	85.95±0.36	107.80±0.38
F(M50)	127.24±0.23	209.54±0.51	227.00±0.53
F(E50)	**140.80±0.1**	**223.98±0.86**	**249.13±0.71**
F(M80)	118.37±0.14	171.68±0.34	193.19±0.21
F(E80)	115.12±0.14	127.60±0.18	190.04±0.53

F(w)-water, F(M50)- 50% methanol; F(E50) -50% ethanol, F(M80) - 80% methanol, F(E80) -80% ethanol

This relation is further investigated with correlation analysis by linear regression between investigated compounds and antioxidant activity determined with DPPH, ABTS and FRAP method in different extraction solvent mixtures. Obtained correlation coefficients are given in Table 3. Very high positive correlations were obtained between antioxidant activity (all three methods) and total phenols (r^2 = 0.9211-0.9794), flavonoids (r^2 = 0.883-0.9762 and r^2 = 0.941-0.9776), phenolic acids (r^2 = 0.8512-0.9947) and proanthocyanidin (r^2 = 0.8702-0.9935) content. Lower correlations were obtained for anthocyanins ranging from r^2 = 0.6197 to r^2 = 0.7801.

Table 3: Correlations between antioxidant activity and phenolic compounds in *P. spinosa* fruit

	DPPH	ABTS	FRAP
	Correlation coefficients r^2		
TP	0.9211	0.9794	0.9415
TF(R)	0.9762	0.8830	0.9497
TF(Q)	0.9410	0.9776	0.9523
TPA	0.9947	0.8512	0.9875
TMA	0.7801	0.6197	0.7452
TPC	0.9935	0.8702	0.9902

Similarly, lower correlations for anthocyanins were reported by Uzelac, *et al.*, 2007 (0.350) and Veličković, *et al.*, 2014 (0.507). Correlations of antioxidant activity and flavonoids were found by Veličković, *et al.*, 2014. Strong contribution of phenolic compounds was also supported by Ruiz-Rodrigez, *et al.*, 2014.

CONCLUSIONS

We can conclude that 50% ethanol is the best solvent for extraction of total phenolic compounds, flavonoids, phenolic acids and proanthocyanidins from blackthorn fruits. Among the solvent mixtures tested, 80% methanol gave the highest yield of anthocyanins. Similarly, according to the content of investigated compounds, antioxidant activity had the highest values for the extract obtained with 50% ethanol and the lowest values of antioxidant activity were determined for the water extract. Strong correlations were observed between content of investigated compounds and antioxidant activity evaluated with DPPH, ABTS and FRAP method. *P. spinosa* fruit can be considered as a valuable natural source of antioxidant compounds which can be found different application in food and pharmaceutical industry.

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Summary/Sažetak

Cilj ovoga rada bila je kvantifikacija fenolnih jedinjenja i određivanje antioksidacijskih svojstava ekstrakata plodova *Prunus spinosa*. Ekstrakcije fenolnih jedinjenja su izvršene sa vodom i četiri vodeno-alkoholne smjese (50% metanolom, 50% etanolom, 80% metanolom i 80% etanolom). Spektrofotometrijsko određivanje fenola provedeno je metodom Folin-Ciocalteu, a flavonoida sa $AlCl_3$ metodom. Arnow reagens je korišten za određivanje sadržaja fenolnih kiselina, dok je sadržaj antocijanina određen pH diferencijalnom metodom. Određivanje proantocijanidina izvršeno je primjenom kiselinsko-butanolne metode. Sadržaj ispitivanih fenolnih jedinjenja kretao se u rasponu od 14,02 do 30,20 mg GAE/g dw (ukupna fenolna jedinjenja), 0,789-1,538 mf RE/g dw i 0,450-1,039 mg QE/g dw (flavonoidi), 4,55-7,24 mg CAE/g dw fenolne kiseline), 0,361-1,05 mg CGE/g dw (antocijanini), 3,97-26,49 mg CE/g dw (proantocijanidini). Najveći udio ispitivanih jedinjenja imao je 50% -tni ekstrakt etanola (izuzev antocijanina) a najniži sadržaj određen je u ekstraktu vode. Najveća antioksidacijska aktivnost određena je za 50%-tni etanolni ekstrakt za sve antioksidacijske metode. Utvrđene su visoke korelacije između antioksidacijske aktivnosti i sadržaja svih analiziranih jedinjenja.