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Research Article

Prunus spinosa L. leaf extracts: polyphenol profile and bioactivities

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Abstract

Prunus spinosa leaf extracts in solvents of different polarity (water, ethanol and acetone), their phenol, flavonoid and anthocyanin contents and biological properties were the object of this study. The richest in phenols as well as in flavonoids was acetone extract with 181.19 mg GAE and 80.10 mg QE per gram of dry extract, respectively. Moreover, the quantity of anthocyanins obtained by HPLC analysis was also the highest in acetone sample. Examined samples possessed antioxidant properties evaluated through four *in vitro* assays (DPPH, ABTS, FRAP and TRC). The acetone extract was proved to be the best antioxidant among tested samples, which could be ascribed to polyphenols, especially anthocyanins. The aqueous and the ethanol extract exhibited antibacterial effects, being particularly active against *B. cereus* and *E. cloacae*. *T. viride*, *P. funiculosum*, *P. ochrochloron*, *P. verrucosum* var. *cyclopium* were the most susceptible among fungal microorganisms examined. Both, the aqueous and the ethanol extract expressed inhibitory activity towards enzymes linked to diabetes mellitus type II. Additionally, the ethanol extract showed significantly higher potential in inhibiting α-glucosidase than the drug used as the positive control. Furthermore, the aqueous sample revealed antitumor effects on following malignant cell lines: HeLa, K562 and MDA-MB-453. The results presented herein suggest that *P. spinosa* leaves should be considered as a natural source of bioactive compounds with potential application in phytopharmacy and food industry.

Keywords: anthocyanins; antioxidant activity; antitumor activity; enzyme-inhibitory activity; leaf extracts; *Prunus spinosa*

Introduction

Plants produce large amounts of phytochemicals with antioxidant abilities to counteract with oxidative stress induced by environmental conditions (Li *et al.*, 2016; Vujanović *et al.*, 2018). Till today, it has been proven that high intake of fruits and vegetables may reduce incidences of serious health disorders caused by oxidative stress, such as neurodegenerative (Tavares *et al.*, 2012), cardiovascular (Kruger *et al.*, 2014), diabetes (He *et al.*, 2019) and cancer (Diaconeasa *et al.*, 2017). Thus, many plant species have been explored for natural

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bioactive compounds that could be implemented in food, pharmacy and cosmetic industry (Cosmulescu *et al.*, 2017). However, wild plant species present easy-accessible, low-cost and rich source of natural bioactive ingredients that is still underexplored and underused.

Genus *Prunus* consists of numerous economically important species which produce edible fruits, such as plums, cherries, peaches, apricots and almonds (Shi *et al.*, 2013). However, it also counts some wild-growing members, for instance, *Prunus spinosa* (blackthorn). Blackthorn or "sloe" is perennial shrub distributed in Northern hemisphere, growing on slopes of wide uncultivated areas, along roads and channels, but also in shelterbelts against the wind (Jovanović, 1972). In folk medicine, blackthorn is reputable as astringent, diuretic, purgative, digestive (Fraternale *et al.*, 2009; Barros *et al.*, 2010), mild laxative (Radovanović *et al.*, 2013), anti-inflammatory and anti-septic agent (Veličković *et al.*, 2014).

P. spinosa is abundant in bioactive polyphenolic compounds, such as phenolic acids, flavonoids, anthocyanins (Radovanović *et al.*, 2013; Veličković *et al.*, 2014; Pozzo *et al.*, 2019), coumarins, nor-isoprenoid glycosides, A-type proanthocyanidins (Kumarasamy *et al.*, 2004). Many authors proved antioxidant effects of blackthorn extracts (Fraternale *et al.*, 2009; Barros *et al.*, 2010; Pinacho *et al.*, 2015; Tahirović *et al.*, 2018; Popović *et al.*, 2020). Moreover, *P. spinosa* and *P. padus* seed extracts possessed antibacterial properties against some pathogenic bacteria (Kumarasamy *et al.*, 2004). Radovanović *et al.* (2013) confirmed the antibacterial effects of *P. spinosa* fruit extracts. Besides pathogenic bacteria, *P. spinosa* ethanol fruit extract also affected the growth of examined microfungi (Veličković *et al.* 2014). Furthermore, *P. spinosa* fruits showed antitumor effects on colorectal cancer cell line (HT29) (Popović *et al.*, 2020). Same authors also showed α-amylase and α-glucosidase inhibitory properties of blackthorn wild genotypes from Serbia.

Taking into account abovementioned publications it could be noted that *Prunus* species, particularly their fruits were recently in focus of research. Nevertheless, available literature data indicate that *P. spinosa* leaves have been neglected and/or underexplored. Therefore, this work was designed to complement current knowledge about blackthorn by extracting phenols and flavonoids from leaves with solvents of different polarities (distilled water, 96% ethanol and acetone). The anthocyanin profile in those extracts was examined and compared. The contribution of polyphenols to antioxidant activity was considered, too. Furthermore, the antibacterial, antifungal, antidiabetic and antitumor activity of aqueous and ethanol extract was determined.

Materials and Methods

Plant material and extract preparation

Leaves were collected from a naturally occurring population of *P. spinosa* L. in Croatia (Brdine; N 44.5936; E 15.6467) in July 2015. The voucher specimen was deposited in the Herbarium of Institute of Botany and Botanical Garden "Jevremovac" (Voucher No. 17482). Plant material was air-dried in shade and grounded into a powder prior to extraction. Pulverised plant material was extracted with different solvents (distilled water, 96% ethanol and acetone) for 24 hours in the dark. The ultrasound was used at the beginning and at the end of extraction for one hour. The extracts were filtered (Whatman filter paper No 1) and the solvents were removed using a rotatory vacuum evaporator (Büchi rotavapor R-114). The obtained dried extracts were kept at +4 °C until further use.

Estimation of total phenol and flavonoid contents

The total phenol content was estimated by their ability to reduce Folin-Ciocalteu reagent (FC) as described by Singleton and Rossi (1965), while the total flavonoid content (TFC) was estimated as previously described by Park *et al.* (1997). The results were measured spectrophotometrically by Perkin Elmer Lambda Bio UV/VIS spectrophotometer and expressed as mg of gallic acid equivalents (GAE) for TPC and as mg of quercetin equivalents (QE) per g of dry weight (DW) for TFC, respectively.

HPLC analyses of anthocyanin profile

To prepare samples of predefined concentration (5 mg mL^{-1}) for HPLC analysis, dry extracts were dissolved in 2N HCl solution in methanol. After 1 hour-incubation in water bath at 90 °C, samples were centrifuged for 15 minutes at 6100 rcf. The supernatant was evaporated till dry by rotatory vacuum evaporator (100 mbar, 40 °C) and redissolved in methanol, filtered through NY filter 0.4 μm and injected into HPLC (Thermo HPLC UltiMate 3000 with UV-DAD (UV-Diode Array Detector)). The aliquots of 15 to 30 μL (10-100 mg mL^{-1}) of the sample were injected in triplicate and separated using AcclaimTM PolarAdvantage II C18 (L=150mm, r=4.6mm, 3μm) column which was kept at constant temperature (30°C). DAD (200-600 nm) was used for detection of anthocyanins in samples. The gradient of following solvents: ddH2O (A), methanol (B) and 1% formic acid in acetonitrile (C), were used as mobile phase with a flow rate of 1mL min^{-1}. The following protocol was applied: isocratic 0-5min (A:B:C=90:0:10), gradient 5-20min (final ratio A:B:C:=0:90:10), isocratic 20-25min (A:B:C=0:90:10). Then, return to initial conditions and isocratic 10 min washout (A:B:C=90:0:10) ensued. The anthocyanins in samples were identified by the comparison of the retention time of unknown peaks with a purchased reference standard (delphinidin, cyanidin, malvidin and pelargonidin) at 525 nm injected under the same chromatographic conditions and by comparison of UV spectra (200-600 nm). The anthocyanin contents were calculated from the six-point calibration curve for each of used reference compounds.

Estimation of antioxidant activity

The antioxidant activity was estimated through DPPH, ABTS, FRAP and TRC *in vitro* colorimetric assays by Perkin Elmer Lambda Bio UV/VIS spectrophotometer as described in our previous work (Veličković *et al.*, 2020).

Estimation of antimicrobial activity

Antibacterial and antifungal activity of *P. spinosa* aqueous and ethanol leaf extracts were estimated as suggested by Soković *et al.* (2010) and Kostić *et al.* (2017), respectively. For that purposes 8 bacterial (*Bacillus cereus* (clinical isolate), *Micrococcus flavus* ATCC10240, *Staphylococccus aureus* ATCC6538, *Listeria monocytogenes* NCTC7973, *Enterobacter cloacae* ATCC35030, *Pseudomonas aeruginosa* ATCC27853, *Salmonella typhimurium* ATCC13311, *Escherichia coli* ATCC35210) and 8 fungal strains (*Aspergillus fumigates* (human isolate), *Aspergillus versicolor* (ATCC11730), *Aspergillus ochraceus* (ATCC12066), *Aspergillus niger* (ATCC6275), *Trichoderma viride* (IAM5061), *Penicillium funiculosum* (ATCC36839), *Penicillium ochrochloron* (ATCC9112), *Penicillium verrucosum* var. *cyclopium* (food isolate) were used.

Determination of antibacterial and antifungal activities was performed in 96 well microtiter plates by serially diluting the samples in Tryptic soy broth and Malt extract broth, respectively. Afterwards, microbial cultures, previously adjusted with sterile saline solution to a concentration of 1×10^5 CFU mL^{-1}, were added to each well, except negative control. Minimal inhibitory concentrations (MICs), the lowest concentrations that caused visible inhibition of bacterial/fungal growth under a binocular microscope, were determined after 24-hours incubation at 37 °C for bacteria and after 72-hours incubation at 28 °C for microfungi. Minimal bactericidal/fungicidal concentrations (MBC/MFCs) were defined as the lowest concentration without visible bacterial/fungal growth indicating 99.5% killing of original inocula. MBCs were determined by re-inoculation of 10 μL of samples into sterile broth and further incubation for 24 hours, while MFCs were determined by re-inoculation of 2 μL of samples into sterile broth and incubation for 72 hours. Ampicillin was used as a positive control for antibacterial and ketoconazole for antifungal activity.

Estimation of enzyme-inhibitory activity

The α-amylase inhibitory activity (α-AIA) was evaluated according to Caraway-Somogyi iodine/potassium method as Zengin *et al.* (2014) reported. Sample solution (25 μL) in different concentrations were mixed with 0.5 mg mL^{-1} α-amylase solution in phosphate buffer (pH 6.8 with 6 mM sodium chloride

(NaCl)) in a final volume of 75 µL in 96-well microtiter plates and pre-incubated for 15 minutes at 37 °C. Then, 50 µL of 0.2% starch solution in phosphate buffer (pH 6.8 with 6mM sodium chloride (NaCl)) was added to initiate the reaction. After 20 minutes of incubation at 37 °C reaction was stopped by adding 25 µL of 1 M hydrochloric acid (HCl). To visualize the reaction, iodine-potassium iodide solution (IKI reagent) was added as colouring agent and absorbance were read at 630 nm by Multiscan Sky Thermo Scientific Finland Plate Reader. The following Equation 1 was used for calculation of inhibited enzyme (%):

$$\text{Percentage of inhibition (\%)} = [(\Delta A_C - \Delta A_S) \times (\Delta A_C)^{-1}] \times 100\% \quad (1)$$

where ΔA_C represents remainder between control solution (containing all reaction reagents except extract) without and with enzyme solution. Similarly, ΔA_S is remainder between sample solutions without and with *α*-amylase.

To estimate *α*-glucosidase inhibitory activity (*α*-GIA) procedure described by Wan *et al.* (2013) was used. Briefly, the mixture of sample solution (120 µL) and 0.6 U mL^{-1} *α*-glucosidase solution in 0.1 M phosphate buffer (pH 6.8) (20 µL) was pre-incubated in 96-well microplates for 15 minutes at 37 °C. The reaction was initiated by adding substrate, 3.5 mM *p*-nitrophenyl-*α*-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) (20 µL) and incubation for 20 minutes at 37 °C. 0.2 M sodium-carbonate (Na_2CO_3) was added to the reaction mixture to stop the reaction. Then, absorbances were read at 405 nm and processed using the same formula (Eq. 1).

The obtained results in both tests were presented through IC_{50} (mg mL^{-1}) values. Glucobay, officially used drug in the treatment of diabetes mellitus type II, which contains acarbose as active compound, was a positive control.

Estimation of antitumor properties

Sample preparation

Stock solutions were prepared by dissolving ethanol crude extract in DMSO and aqueous in distilled water in a final volume of 20 mg mL^{-1}. Samples were obtained by dilution of stock solution in a complete nutrient medium (RPMI-1640 without phenol red) supplemented with 3 mM L-glutamine, 100 µg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin, 10% heat-inactivated fetal bovine serum (FBS), and 25 mM Hepes and adjusted to pH 7.2 by bicarbonate solution.

Cell culture

The human cervical carcinoma (HeLa cells), human breast cancer (MDA-MB-453) and human chronic myelogenous leukaemia (K562) malignant cell lines were used for examination of antitumor activity. Examined tumour cell lines were grown in a monolayer at 37 °C in a humidified air atmosphere with 5% CO_2, except K562 cells which were cultured in a suspension in the complete nutrient medium. Human embryonic lung fibroblast (MRC-5), a non-cancerous cell line was used as control.

Treatment of cancerous and control cell lines

Five different concentration of the sample (ranging from 0.125 to 2 mg/mL) were added to 96-well microtiter plates where, 20 hours before, Hela (2,500 cells per well), MDA-MB-453 (3,000 cells per well) an MRC-5 (5,000 cells per well) cells were seeded. Two hours prior to the addition of the examined sample, K562 cells were seeded at 5,000 per well to give desired final concentrations within the range mentioned above. Blank was a nutrient medium containing an adequate concentration of extract without seeded cells.

Estimation of cell survival

The cytotoxic effects of examined samples on malignant and control cell lines were estimated by the microculture tetrazolium test (MTT) described by Mosmann (1983) with modification according to Ohno and Abe (1991). In short, cultures were incubated with examined sample and 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline (MTT dye solution) for

another 4 hours at 37 °C in a humidified atmosphere of 5% CO_2 (v/v). Subsequently, 100 µL of 10% sodium dodecyl sulfate (SDS) was added to visualize the activity of viable cells by extracting insoluble formazan, the product of MTT dye conversion by viable cells. The absorbance was read at 570 nm after 24 hours and cell survival (S%) was calculated by the following Equation 2:

$$S(\%) = (A_S \times A_C^{-1}) \times 100(\%) \quad (2)$$

where A_S and A_C represent the absorbance of cells grown in the presence of examined sample and cells grown in nutrient medium only, respectively. The absorbance of blank was subtracted from the corresponding sample incubated with target cells.

The results were expressed through IC_{50} values which represent the extract concentration that causes a 50% decrease in the number of survived malignant and normal cells.

Statistical analysis

The results were obtained from three independent experiments and expressed as their average value (AV) ± standard error (SE). Correlations among phenols, flavonoids, anthocyanins and antioxidant activities were presented through Pearsons' coefficient of correlation and interpreted according to Taylor (1990). Results were processed by MS Office Excel 2007.

Results

The yield of *P. spinosa* leaf extracts ranged from 4.36% for acetone to 13.65% for aqueous extract (Table 1). The richest in phenols, as well as in flavonoids was acetone leaf extract with 181.00 mg GAE g^{-1} DW and 80.10 mg QE g^{-1} DW, respectively (Table 1).

Table 1. Yields (%), total phenol (TPC) and total flavonoid contents (TFC) in different *P. spinosa* leaf extracts

Sample	Yield (%)	TPC (mg GAE g^{-1})	TFC (mg QE g^{-1})
Water	13.65	142.40±3.82	36.28±0.41
Ethanol	9.14	116.63±1.62	45.52±0.90
Acetone	4.36	181.19±1.70	80.10±0.00

HPLC analysis enabled the identification and quantification of four basic anthocyanins: delphinidin (Dp), cyanidin (Cy), malvidin (Mv) and pelargonidin (Pg). Similar to TPC and TFC the sum of identified anthocyanins was the highest in acetone extract (4.81 mg g^{-1} of DW) (Table 2). The cyanidin was dominant anthocyanin compound in all examined samples (Figure 1).

Table 2. The anthocyanin compounds in *P. spinosa* leaf extracts and their sum (TAC)

Sample	Dp (mg g^{-1})	Cy (mg g^{-1})	Mv (mg g^{-1})	Pg (mg g^{-1})	TAC (mg g^{-1})
Water	0.07	0.74	0.27	0.05	1.13
Ethanol	0.08	1.30	0.69	0.17	2.24
Acetone	0.08	2.78	1.46	0.49	4.81

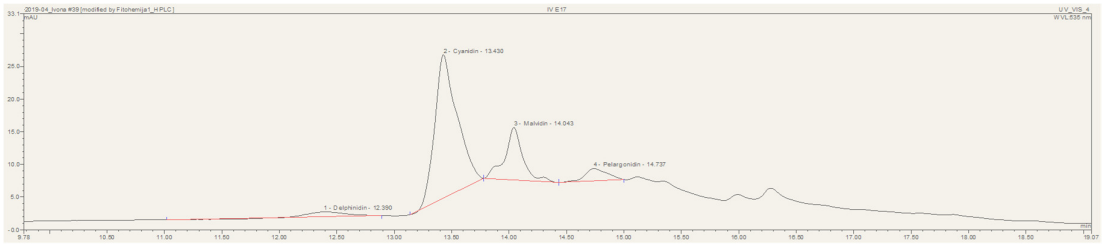

Figure 1. HPLC chromatogram of *P. spinosa* leaf extract
Peak identification: 1) Delphinidin; 2) Cyanidin; 3) Malvidin; 4) Pelargonidin

The acetone extract was the most effective in scavenging DPPH and ABTS free radicals with EC_{50} values 44.57 and 16.12 mg mL^{-1}, respectively. The values obtained from FRAP assay varied among 0.52 and 0.80 µmol Fe^{2+} equivalents per g of DW being the highest for the acetone extract. Additionally, the acetone extract revealed the best antioxidant activity through TRC assays, too (Table 3).

Table 3. Antioxidant activities of *P. spinosa* leaf extracts estimated by four *in vitro* colorimetric methods

Sample	DPPH[1]	ABTS[1]	FRAP[2]	TRC[1]
Water	65.84±0.64	21.04±0.53	0.52±0.01	1,422.00±3.33
Ethanol	57.07±2.77	22.74±0.13	0.62±0.03	1,040.00±0.38
Acetone	44.57±0.73	16.12±0.20	0.80±0.00	633.05±11.02
BHA	**5.43±0.01**	**NM**	**1.83±0.24**	**10.97±0.17**
***L*-ascorbic acid**	**3.74±0.07**	**2.33±0.07**	**6.30±0.13**	**8.72±0.48**

[1]DPPH and ABTS free radical scavenging antioxidant activities, as well as total reducing capacity (TRC) expressed in terms of EC_{50} values (µg mL^{-1}); [2]Ferric Reducing Antioxidant Power expressed in µmol Fe^{+2} equivalents per g of dry extract; NM not measured

The high positive correlation was found between phenols and flavonoids, phenols and anthocyanins, as well as between flavonoids and anthocyanins (Table 4).

The strong negative correlation was established among phenols, flavonoids and anthocyanins and values obtained for DPPH, ABTS and TRC assays. However, they positively correlated to FRAP values. The values of *r* obtained for the relation of free radical scavenging assays and TRC values indicated high positive correlation. Nevertheless, FRAP assay strongly negatively correlated with DPPH, ABTS and TRC with r values -0.9980, -0.8197 and -0.9892, respectively.

Table 4. Correlations between TPC, TFC, antioxidant and antidiabetic properties of *P. spinosa* leaf extracts expressed through Pearson's coefficient of correlation (*r*)

R	TPC	TFC	TAC	DPPH	ABTS	FRAP	TRC
TPC	1						
TFC	0.8202	1					
TAC	0.7609	0.995	1				
DPPH	-0.6746	-0.9756	-0.9923	1			
ABTS	0.9876	-0.8999	-0.8535	0.7822	1		
FRAP	0.7194	0.9874	0.9981	-0.9980	-0.8197	1	
TRC	-0.6102	-0.9537	-0.9784	0.9964	0.7272	0.9892	1

r ≤ 0.35 weak correlation; 0.36 < r < 0.67 moderate correlation; 0.68 < r < 1 strong correlation according to Taylor, 1990

The results of antibacterial activity were presented in Table 5. MIC values varied from 1.42 to 22.73 mg mL^{-1}, while MBC from 2.84 to 45.45 mg mL^{-1}. The examined samples showed lower inhibitory activity towards tested bacteria than commercial drug ampicillin. The ethanol extract was more efficient against examined pathogenic bacteria, particularly towards *E. cloacae* and *B. cereus*.

Table 5. Antibacterial activity of *P. spinosa* leaf extracts determined by microdilution method expressed through MIC/MBC values

Sample	Water		Ethanol		**Ampicillin**	
	MIC[1]	MBC[2]	MIC[1]	MBC[2]	**MIC[1]**	**MBC[2]**
Test bacterial microorganism						
Gram$^+$ bacteria						
Bacillus cereus (clinical isolate)	5.68	11.36	2.84	5.68	**0.17**	**0.20**
Micrococcus flavus ATCC10240	22.73	45.45	11.36	22.73	**0.13**	**0.15**
Staphylococcus aureus ATCC6538	22.73	45.45	11.36	22.73	**0.10**	**0.20**
Listeria monocytogenes NCTC7973	22.73	45.45	11.36	22.73	**0.20**	**0.33**
Gram$^-$ bacteria						
Enterobacter cloacae ATCC35030	5.68	11.36	1.42	2.84	**0.17**	**0.20**
Pseudomonas aeruginosa ATCC27853	11.36	22.73	22.73	45.45	**0.40**	**0.67**
Salmonella typhimurium ATCC13311	22.73	45.45	22.73	45.45	**0.13**	**0.20**
Escherichia coli ATCC35210	22.73	45.45	22.73	45.45	**0.18**	**0.27**

[1,2] Minimal inhibitory (MICs) and bactericidal (MBCs) concentrations in mg mL^{-1}

In the antifungal assay the MIC and MFC values ranged from 2.74 to 23.15 mg mL^{-1} and from 5.48 to 46.30 mg mL^{-1}, respectively (Table 6). In contrast to antibacterial activity, the aqueous sample showed slightly better antimycotic properties, but still lower than the positive control (ketoconazole). *T. viride*, *P. funiculosum*, *P. ochrochloron* were susceptible to both tested extracts, while *A. ochraceus* and *P. verrucosum* var *cyclopium* were efficiently inhibited by the aqueous extract.

Table 6. Antifungal activity of *P. spinosa* leaf extracts determined by microdilution method expressed through MIC/MFC values

Sample	Water		Ethanol		**Ketoconazole**	
	MIC[1]	MFC[2]	MIC[1]	MFC[2]	**MIC[1]**	**MFC[2]**
Test fungal microorganism						
Aspergillus fumigatus (human isolate)	23.15	46.30	11.57	23.15	**0.23**	**0.67**
Aspergillus versicolor ATCC11730	11.06	22.12	22.12	44.25	**0.20**	**0.47**
Aspergillus ochraceus ATCC12066	5.73	11.47	11.47	22.94	**0.20**	**0.27**
Aspergillus niger ATCC6275	11.26	22.52	22.52	45.04	**0.27**	**0.42**
Trichoderma viride IAM5061	2.87	5.73	5.74	11.47	**0.83**	**2.00**

Penicilliumfuniculosum ATCC36839	5.68	11.36	2.84	5.68	**0.23**	**0.67**
Penicillium ochrochloron ATCC9112	5.48	10.96	2.74	5.48	**1.33**	**1.67**
Penicillium verrucosum var. *cyclopium* (food isolate)	5.58	11.16	11.16	22.32	**0.27**	**0.40**

[1,2] Minimal inhibitory (MICs) and bactericidal (MFCs) concentrations in mg mL^{-1}

The enzyme-inhibitory activity was evaluated through the ability of extracts to inhibit α-amylase and α-glucosidase, diabetes-linked enzymes and results were shown in Table 7. Both extracts were more potent inhibitors of α-glucosidase. Furthermore, the ethanol sample revealed α-glucosidase inhibitory activity (α-GIA) importantly higher even than Glucobay, officially used medicine in the treatment of diabetes mellitus type II.

Table 7. *In vitro* evaluation of enzyme-inhibitory and antitumor activity of *P. spinosa* leaf extracts

	Enzyme-inhibitory activity		Antitumor activity			
	α-AIA[1]	α-GIA[2]	HeLa[3]	K562[3]	MDA-MB-453[3]	MRC-5[3]
Sample						
Water	81.98±1.73	2.95±0.00	770.00±4.24	865.00±9.90	877.00±39.60	1,244.00±43.84
Ethanol	10.86±0.08	0.03±0.01	>2,000	>2,000	>2,000	>2,000
Glucobay	**0.20±0.01**	**0.23±0.02**				

[1]α-Amylase inhibitory activity expressed through IC_{50} values (mg mL^{-1}); [2]α-Glucosidase inhibitory activity expressed through IC_{50} values (mg mL^{-1}); [3]antitumor activity on HeLa, K562 and MDA-MB-453 human carcinoma cell lines and MRC-5 control cell line, expressed through IC_{50} (μg mL^{-1})

The results of antitumor activity presented in Table 7. indicate that the aqueous sample decreased cell survival of HeLa, K562 and MDA-MB-453 tumour cell lines, but also showed selectivity in effects on malignant and healthy cells. On the other hand, the ethanol sample had no antitumor properties.

Discussion

Extraction conditions and techniques influence on extraction yields, polyphenol profile and bioactivity of extracts. Novel extraction methods including ultrasound-assisted extraction (UAE), which is used in this work, are time-consuming and more efficient in extracting bioactive compounds from solid plant matrixes than older traditionally used techniques (Castro-Lόpez *et al.*, 2017). In this work, the almost three times higher yield was found in the aqueous than in the acetone extract (Table 1). The authors who previously examined extraction yields of *Prunus* spp. reported slightly higher yields of the ethanol than the aqueous leaf extract which could be ascribed to the differences among used extraction procedures (Sahan *et al.*, 2011). Despite the lowest yield, the acetone extract was the richest in phenols, flavonoids and anthocyanins. Earlier, several authors quantified phenols in *P. spinosa* fruits and found a few times lower values than those presented herein (Radovanović *et al.*, 2013; Veličković *et al.*, 2014; Popović *et al.*, 2020). This is probably due to the differences among plant organs used for extraction. On the other hand, Park *et al.* (2012) examined phenol content in different organs of *Prunus* spp. and for leaf extract found values close to the ones determined in this study (121.41 mg GAE g^{-1} DW). Moreover, results for TPC for *Prunus* spp. leaf extract reported by Karabegović *et al.* (2014) varied from 85 to 119.4 mg GAE g^{-1} DW. In the same work, TFC values were in accordance with our results. Furthermore, Pinacho *et al.* (2015), evaluated TPC and TFC extracted from *P. spinosa* branches, leaves and fruits using dichloromethane, ethyl-acetate, ethanol and water extract. The results for leaf extract demonstrated in their work ranged from 38.57 to 228.56 mg GAE g^{-1} DW for TPC and from <0.01 to 196.88 mg RE g^{-1} DW for TFC. HPLC analysis of *P. spinosa* leaf extracts enabled the identification of four

anthocyanin compounds (delphinidin, cyanidin, malvidin and pelargonidin) and quantification of their contents. According to available literature data, there is a lack of information on HPLC analysis of *P. spinosa* leaf extracts with only a few reports about compounds found in blackthorn fruits, branches or flowers. For example, Mechini *et al.* (2017) previously reported lower amounts of summarized anthocyanin compounds in blackthorn fruits, while Popović *et al.* (2020) obtained results congruent with ours. Veličković *et al.* (2014) performed HPLC analysis of blackthorn fruit extracts and in aqueous extract identified anthocyanin compounds (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside and peonidin-3-*O*-glucoside), while in ethanol and ethanol-aqueous extract phenolic acids (neochlorogenic and caffeic acid) and flavonoids (myricetin and quercetin) were also present. Similar results were also reported by Popović *et al.* (2020), recently. Pinacho *et al.* (2015) examined polyphenol profile of *P. spinosa* branches before and after *in vitro* digestion and besides phenolic acids found coumarins, flavonols and proanthocyanidins. Varga *et al.* (2017) detected phenolic acids and quercetin glycosides in *P. spinosa* leaves. Additionally, Owczarek *et al.* (2016) also identified phenolic acids, quercetin and kaempferol glycosides in blackthorn leaves.

The strong positive correlation was established among TPC and TFC/TAC, as well as among TFC and TAC. Similarly, Pinacho *et al.* (2015) confirmed that main phenol compounds were flavonoids. Moreover, phenols highly correlated with identified anthocyanin compounds in blackthorn fruits from North Serbia (Popović *et al.*, 2020).

The correlations found between phenols, flavonoids and anthocyanins concentrations and values obtained for DPPH, ABTS, FRAP and TRC suggest that polyphenols, particularly anthocyanins are probably major contributors to antioxidant properties of blackthorn leaves. That was previously confirmed by several researchers (Barros *et al.*, 2010; Radovanović *et al.*, 2013; Veličković *et al.*, 2014; Pinacho *et al.*, 2015; Popović *et al.*, 2020). Furthermore, a high correlation among DPPH and FRAP/TRC values indicate that antioxidant compounds found in blackthorn leaf extracts react rather by electron than hydrogen atom transfer mechanism (Popović *et al.*, 2020).

The aqueous and ethanol leaf extracts of *P. spinosa* were active against examined bacterial and fungal strains. The most susceptible bacterial strains were *E. cloacae* and *B. cereus*, while *T. viride*, *P. funiculosum*, *P. ochrochloron* were among the most affected fungal pathogens. According to Kumarasamy *et al.*, (2004), *P. spinosa* methanol extract of seed was effective against *Lactobacillus plantarum*, *S. aureus* and *Citrobacter freundii*. Radovanović *et al.* (2013) and Veličković *et al.* (2014) confirmed the antibacterial effects of *P. spinosa* fruits on several bacterial strains including *S. aureus*, *E. coli*, *P. aeruginosa* and *M. flavus*. Blackthorn aqueous fruit extract was also active against *S. aureus*, *Streptococcus* sp. and *Escherichia coli* (Gegiu *et al.*, 2015).

According to the available literature data, this is the first report regarding anti-diabetic properties of blackthorn leaf extracts. Presented results indicate that examined samples possessed notable potential in inhibiting α-amylase and α-glucosidase, enzymes linked with diabetes mellitus type II. It could be also noticed that the ethanol sample (0.03 mg mL^{-1}) exhibited significantly higher α-GIA than the positive control (0.23 mg mL^{-1}). Additionally, results presented herein were congruent with results obtained for blackthorn fruit extracts by Popović *et al.* (2020).

Furthermore, the antitumor properties of blackthorn leaf extracts have not been examined previously. According to presented results, the ethanol extract showed effects on following malignant human cell lines: HeLa, K562, MDA-MB-453. Similarly, to anti-diabetic properties, some researchers previously confirmed antitumor properties of *P. spinosa* fruit extracts (Karakas *et al.*, 2019; Popović *et al.*, 2020).

Conclusions

The blackthorn leaf extracts are rich in phenols, flavonoids and anthocyanins which highly correlated with its antioxidant activity. Among the examined extracts the acetone extract was the most potent antioxidant possibly due to the highest content of polyphenol compounds, especially anthocyanins found in this extract.

The obtained results indicated that the mentioned leaf extracts exhibited antimicrobial, antidiabetic and antitumor effects. It should be noted that the ethanol sample was particularly effective in inhibiting α-glucosidase, carbohydrate hydrolysing enzyme, with significantly lower IC50 value than the positive control (Glucobay). Undoubtedly, *P. spinosa* leaves were unduly treated as waste. Rather it should be observed as an easily accessible natural source of bioactive compounds with potential application in food supplementation and phytopharmacy. Thus, further examination of blackthorn anthocyanins and their bioactivities should be performed.

Authors' Contributions

Investigation: IV, ŽŽ, NR; Methodology: IV, ŽŽ, MI, NR; Supervision: MS, SG; Writing-original draft: IV; Writing-review and editing: PDM, SG; All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Barros L, Carvalho AM, Morais JS, Ferreira IC (2010). Strawberry-tree, blackthorn and rose fruits: Detailed characterisation in nutrients and phytochemicals with antioxidant properties. Food Chemistry 120(1):247-254. *https://doi.org/10.1016/j.foodchem.2009.10.016*

Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Analytical Biochemistry 239:70-76. *https://www.sciencedirect.com/science/article/abs/pii/S0003269796902924*

Blois MS (1958). Antioxidant determination by use of stable free radical. Nature 181:1199-1200. *https://doi.org/10.1038/1811199a0*

Castro-López C, Ventura-Sobrevilla JM, González-Hernández MD, Rojas R, Ascacio-Valdés JA, Aquilar CN, Martínez-Ávila GCG (2017). Impact of extraction techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts. Food Chemistry 237:1139-1148. *https://doi.org/10.1016/j.foodchem.2017.06.032*

Cosmulescu S, Trandafir I, Nour, V (2017). Phenolic acids and flavonoids profile of extracts from edible wild fruit and their antioxidant properties. International Journal of Food Properties 20(12):3124-3134. *https://doi.org/10.1080/10942912.2016.1274906*

Diaconeasa Z, Ayvaz H, Rugina D, Leopold L, Stanila A, Socaciu C, ... Jefferson A (2017). Melanoma inhibition by anthocyanins is associated with the reduction of oxidative stress biomarkers and changes in mitochondrial membrane potential. Plant Foods for Human Nutrition 72(4):404-410. *https://doi.org/10.1007/s11130-017-0638-x*

Fraternale D, Giamperi L, Bucchini A, Sestili P, Paolillo M, Ricci D (2009). *Prunus spinosa* fresh fruit juice: antioxidant activity in cell-free and cellular systems. Natural product communications 4(12):1934578X0900401211. *https://doi.org/10.1177/1934578X0900401211*

Gegiu G, Branza AD, Bucur L, Grigorian M, Tache T, Badea V (2015). Contributions to antimicrobial and antifungal study of *Prunus spinosa* L. Farmacia 63(2):275-279. *http://www.revistafarmacia.ro/201502/art-18-Gegiu_275-279.pdf*

He JH, Chen LX, Li H (2019). Progress in the discovery of naturally occurring anti-diabetic drugs and in identification of their molecular targets. Fitoterapia 134:270-289. *https://doi.org/10.1016/j.fitote.2019.02.033*

Hussain G, Rasul A, Anwar H, Aziz N, Razzaq A, Wei W, ... Li X (2018). Role of plant derived alkaloids and their mechanism in neurodegenerative disorders. International Journal of Biological Sciences 14(3):341-357. *https://doi.org/10.7150/ijbs.23247*

Jovanović B (1972). *Prunus* L. In: Tatić B, Josifović M, Stjepanović L, Janković MM, Gajić M, Kojić M, Diklić N (Eds). Flora S.R. Srbije, Vol 4. SANU, Beograd, Srbija pp 179.

Karabegović IT, Stojičević SS, Veličković DT, Todorović ZB, Nikolić NČ, Lazić ML (2014). The effect of different extraction techniques on the composition and antioxidant activity of cherry laurel (*Prunus laurocerasus*) leaf and fruit extracts. Industrial Crops and Products 54(2014):142-148. *https://doi.org/10.1016/j.indcrop.2013.12.047*

Karakas N, Okur ME, Oztruk I, Ayla S, Karadag AE, Polat DÇ (2019). Antioxidant activity of blackthorn (*Prunus spinosa* L.) fruit extract and cytotoxic effects on various cancer cell lines. Medeniyet Medical Journal 34:297-304. *https://doi.org/10.5222/MMJ.2019.87864*

Kostić M, Smiljković M, Petrović J, Glamočlija J, Barros L, Ferreira IC, ... Soković M (2017). Chemical, nutritive composition and a wide range of bioactive properties of honey mushroom *Armillaria mellea* (Vahl: Fr.) Kummer. Food & Function 8(9):3239-3249. *https://doi.org/10.1039/C7FO00887B*

Kruger MJ, Davies N, Myburgh KH, Lecour S (2014). Proanthocyanidins, anthocyanins and cardiovascular diseases. Food Research International 59:41-52. *https://doi.org/10.1016/j.foodres.2014.01.046*

Kumarasamy Y, Cox PJ, Jaspars M, Nahar L, Sarker SD (2004). Comparative studies on biological activities of *Prunus padus* and *P. spinosa*. Fitoterapia 75(1):77-80. *https://doi.org/10.1016/j.fitote.2003.08.011*

Lee S, Jun W (2001). A phylogenetic analysis of *Prunus* and the Amygdaloideae (Rosaceae) using ITS sequences of nuclear ribosomal DNA. American Journal of Botany 88(1):150-160. *https://doi.org/10.2307/2657135*

Li H, Tsao R, Deng Z (2012). Factors affecting antioxidant potential and health benefits of plant foods. Canadian Journal of Plant Science 92:1101-1111. *https://doi.org/10.4141/cjps2011-239*

Meschini S, Pellegrini E, Condello M, Occhionero G, Delfine S, Condello G, Mastrodonato F (2017). Cytotoxic and apoptotic activities of *Prunus spinosa* Trigno ecotype extract on human cancer cells. Molecules 22(9):1578. *https://doi.org/10.3390/molecules22091578*

Miller N, Rice-Evans C (1997). Factors influencing the antioxidant activity determined by the ABTS radical cation assay. Free Radical Research 26:195-199. *https://doi.org/10.3109/10715769709097799*

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65:55-63.

Ohno M, Abe T (1991). Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). Journal of Immunological Methods 145:199-203. *https://doi.org/10.1016/0022-1759(91)90327-C*

Owczarek A, Magiera A, Matczak M, Piotrowska DG, Olszewska MA, Marchelak A (2016). Optimisation of preparative HPLC separation of four isomeric kaemferol diglycosides from *Prunus spinosa* L. by application of the response surface methodology. Phytochemistry Letters 20:415-424. *https://doi.org/10.1016/j.phytol.2017.01.010*

Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition 44:307-315. *https://doi.org/10.5264/eiyogakuzashi.44.307*

Park JW, Yuk HG, Lee SC (2012). Antioxidant and tyrosinase inhibitory activities of different parts of oriental cherry (*Prunus serrulata* var *spontonea*). Food Science and Biotechnology 21(2):339-343. *https://doi.org/10.1007/s10068-012-0045-x*

Park K, Koo MH, Ikegaki M, Contado JLM (1997). Comparison of the flavonoid aglycone contents of *Apis melifera* propolis from various regions of Brazil. Arquivos de Biologia e Tecnologia 40(1):97-106.

Pinacho R, Cavero RY, Astiasarán I, Ansorena D, Calvo MI (2015). Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of *in vitro* digestion on their antioxidant capacity. Journal of Functional Foods 19:49-62. *https://doi.org/10.1016/j.jff.2015.09.015*

Popović BM, Blagojević B, Pavlović RŽ, Mićić N, Bijelić S, Bogdanović B, ... Serra AT (2020). Comparison between polyphenol profile and bioactive response in blackthorn (*Prunus spinosa* L.) genotypes from north Serbia-from raw data to PCA analysis. Food Chemistry 302:125373. *https://doi.org/10.1016/j.foodchem.2019.125373*

Radovanović BC, Anđelković SM, Radovanović AB, Anđelković MZ (2013). Antioxidant and antimicrobial activity of polyphenol extracts from wild berry fruits grown in southeast Serbia. Tropical Journal of Pharmaceutical Research 12(5):813-819. *http://dx.doi.org/10.4314/tjpr.v12i5.23*

Sahan Y (2011). Effect of *Prunus laurocerasus* L. (cherry laurel) leaf extracts on growth of bread spoilage fungi. Bulgarian Journal of Agricultural Science 17(1):83-92. *https://www.cabdirect.org/cabdirect/abstract/20113221549*

Shi S, Li J, Sun J, Yu J, Zhou S (2013). Phylogeny and classification of *Prunus sensu lato* (Rosaceae). Journal of Integrative Plant Biology 55(11):1069-1076. *https://doi.org/10.1111/jipb.12095*

Singleton VJ, Rossi JA (1965). Colometry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16:144-158. *https://www.ajevonline.org/content/16/3/144*

Soković M, Glamočlija J, Marin PD, Brkić D, Van Griensven LJ (2010). Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an *in vitro* model. Molecules 15(11):7532-7546. *https://doi.org/10.3390/molecules15117532*

Tahirovic A, Basic N, Copra-Janicijevic A (2018). Effect of solvents on phenolic compounds extraction and antioxidant activity of *Prunus spinosa* L. fruits. Glasnik hemicara i tehnologa Bosne i Hercegovine (50):19-24. *http://www.pmf.unsa.ba/hemija/glasnik/files/Issue%2050/5-19-24-Tahirovi_A.pdf*

Tavares L, Figueira I, Macedo D, McDougall GJ, Leitão MC, Vieira HLA, ... Santos CN (2012). Neuroprotective effect of blackberry (*Rubus* sp.) polyphenols is potentiated after simulated gastrointestinal digestion. Food Chemistry 131:1443-1452. *https://doi.org/10.1016/j.foodchem.2011.10.025*

Taylor R (1990). Interpretation of the correlation coefficient: a basic review. Journal of Diagnostic Medical Sonography 6(1):35-39. *https://doi.org/10.1177/875647939000600106*

Varga E, Domokos E, Fogarasi E, Steanesu R, Fülöp I, Croitoru MD, Laszkó-Zöld E (2017). Polyphenolic compounds analysis and antioxidant activity in fruits of *Prunus spinosa* L. Acta Pharmaceutica Hungarica 87(1):19-25. *https://pubmed.ncbi.nlm.nih.gov/29489094/*

Veličković I, Žižak Ž, Rajčević N, Ivanov M, Soković M, Marin P, Grujić S (2019). Examination of the polyphenol content and bioactivities of *Prunus spinosa* L. fruit extracts. Archives of Biological Sciences 72(1):105-115. *https://doi.org/10.2298/ABS191217004V.*

Veličković JM, Kostić DA, Stojanović GS, Mitić SS, Mitić MN, Ranđelović SS, Đorđević AS (2014). Phenolic composition, antioxidant and antimicrobial activity of the extracts from *Prunus spinosa* L. fruit. Hemijska Industrija 68(3):297-303. *https://doi.org/10.2298/HEMIND130312054V*

Vujanović M, Zengin G, Đurović S, Mašković P, Cvetanović A, Radojković M (2019). Biological activity of extracts of traditional wild medicinal plants from the Balkan Peninsula. South African Journal of Botany 120:213-218. *https://doi.org/10.1016/j.sajb.2018.06.012*

Wan LS, Min QX, Wang YL, Yue YD, Chen JC (2013). Xanthone glycoside constituents of *Swertia kouitchensis* with *α*-glucosidase inhibitory activity. Journal of Natural Products 76(7):1248-1253. *https://doi.org/10.1021/np400082g*

Zengin G, Sarikurkcu C, Aktumsek A, Ceylan R, Ceylan O (2014). A comprehensive study on phytochemical characterization of *Haplophyllum myrtifolium* Boiss. endemic to Turkey and its inhibitory potential against key enzymes involved in Alzheimer, skin diseases and type II diabetes. Industrial Crops and Products 53:244-251. *https://doi.org/10.1016/j.indcrop.2013.12.043*