**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
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 (Velićković et al., 2014).

*P. spinosa* is abundant in bioactive polyphenolic compounds, such as phenolic acids, flavonoids, anthocyanins (Radovanović et al., 2013; Velićković 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 (Velićković 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 ground 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 (Velicković 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, *Staphylococcus 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 (%):

\[ \text{Percentage of inhibition} \% = \left( \frac{\Delta A_{\text{C}} - \Delta A_{\text{S}}}{\Delta A_{\text{C}}} \right) \times 100\% \]  

(1)

where \( \Delta A_{\text{C}} \) represents remainder between control solution (containing all reaction reagents except extract) without and with enzyme solution. Similarly, \( \Delta A_{\text{S}} \) 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) 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\(_2\)CO\(_3\)) 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<sub>2</sub> (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:

\[ S(\%) = \frac{A_s - A_c}{A_c} \times 100(\%) \] (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<sub>50</sub> 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<sup>-1</sup> DW and 80.10 mg QE g<sup>-1</sup> DW, respectively (Table 1).

| Sample   | Yield (%) | TPC (mg GAE g<sup>-1</sup>) | TFC (mg QE g<sup>-1</sup>) |
|----------|-----------|------------------------------|-----------------------------|
| 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<sup>-1</sup> of DW) (Table 2). The cyanidin was dominant anthocyanin compound in all examined samples (Figure 1).

| Sample   | Dp (mg g<sup>-1</sup>) | Cy (mg g<sup>-1</sup>) | Mv (mg g<sup>-1</sup>) | Pg (mg g<sup>-1</sup>) | TAC (mg g<sup>-1</sup>) |
|----------|------------------------|------------------------|------------------------|------------------------|-------------------------|
| 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                    |
The acetone extract was the most effective in scavenging DPPH and ABTS free radicals with EC\textsubscript{50} values 44.57 and 16.12 mg mL\textsuperscript{-1}, respectively. The values obtained from FRAP assay varied among 0.52 and 0.80 μmol Fe\textsuperscript{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\textsuperscript{1} | ABTS\textsuperscript{1} | FRAP\textsuperscript{2} | TRC\textsuperscript{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 |

\textsuperscript{1}DPPH and ABTS free radical scavenging antioxidant activities, as well as total reducing capacity (TRC) expressed in terms of EC\textsubscript{50} values (µg mL\textsuperscript{-1}); \textsuperscript{2}Ferric Reducing Antioxidant Power expressed in μmol Fe\textsuperscript{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 anti diabetic 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   |

\( r \leq 0.35 \) 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 MIC¹ | Water MBC² | Ethanol MIC¹ | Ethanol MBC² | Ampicillin MIC¹ | Ampicillin MBC² |
|---------------------------------|------------|------------|--------------|--------------|----------------|----------------|
| **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 MIC¹ | Water MFC² | Ethanol MIC¹ | Ethanol MFC² | Ketoconazole MIC¹ | Ketoconazole MFC² |
|---------------------------------|------------|------------|--------------|--------------|-------------------|-------------------|
| **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              |
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

| Sample          | α-AIA¹ | α-GIA² | HeLa² | K562² | MDA-MB-453² | MRC-5² |
|-----------------|--------|--------|-------|-------|-------------|-------|
| 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 |       |       |             |       |

¹α-Amylase inhibitory activity expressed through IC₅₀ values (mg mL⁻¹); ²α-Glucosidase inhibitory activity expressed through IC₅₀ values (mg mL⁻¹); ³antitumor activity on HeLa, K562 and MDA-MB-453 human carcinoma cell lines and MRC-5 control cell line, expressed through IC₅₀ (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-López 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*, *S. pyogenes* 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.

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