Examination of the polyphenol content and bioactivities of *Prunus spinosa* L. fruit extracts

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**Abstract:** We investigated the total phenolic and flavonoid contents and the anthocyanin profiles in aqueous, ethanol and acetone extracts of *Prunus spinosa* (Rosaceae) fruit, and their antioxidant, antibacterial, antifungal, antidiabetic and antitumor properties. The contribution of polyphenol contents to the bioactivity of the extracts was calculated and observed through Pearson's coefficient of correlation. The acetone extract was the richest in phenols and anthocyanins and the ethanol extract in flavonoids. Cyanidin was the most abundant anthocyanin compound in all examined extracts. The ethanol extract showed the most promising antioxidant activity in DPPH, ABTS and FRAP assays. Tested bacteria were more affected by the ethanol than by the aqueous extract. Both the ethanol and aqueous extracts exhibited potential antidiabetic effects, observed as inhibition of β-amylase and β-glucosidase, enzymes linked with diabetes mellitus type II. The ethanol extract was a potent β-glucosidase-inhibitor with a significantly lower IC50 value than the positive control, glucobay, used to treat diabetes mellitus type II. Neither the ethanol nor the aqueous extracts had any effects on tested human malignant cell lines. Our results indicate that the ethanol extract showed the most pronounced *in vitro* antioxidant and antimicrobial effects, and a potential antidiabetic activity, which can be ascribed to its high flavonoid content. Our results indicate that research of compounds, particularly of flavonoids present in the ethanol extract and their anti-diabetic properties should be examined further.

**Keywords:** *P. spinosa* (blackthorn); antioxidant activity; antimicrobial activity; antidiabetic activity; β-glucosidase inhibitory activity

**INTRODUCTION**

The genus *Prunus* L. (Rosaceae) includes about 200 species that are widespread in the temperate climate of the northern hemisphere, particularly in East Asia [1]. *Prunus spinosa* L., also known as blackthorn or sloe, is a deciduous, dense shrub up to 4 m in height, which grows wild on uncultivated areas, beside roads and canals. *P. spinosa* stems are usually covered by thorns. Fruits are spherical bluish-black with a single stone and a sour and pungent taste [2,3]. *Prunus* spp. contain many economically important species whose fruits are freshly consumed, frozen and processed [4,5]. Most of them possess laxative, diuretic, diaphoretic, astringent [6-11], spasmylytic [12] and analgesic properties [8,10-11,13], and are used in folk medicine for the treatment of common colds and cough [14], diabetes, obesity, kidney stones [13], nephritis, respiratory [15,16] and cardiovascular disorders [15]. *Prunus* species, like other plants with intensely- and dark-colored fruits, are considered to be a treasury of health-promoting polyphenols, particularly anthocyanins [17], phenolic acids [18-20], flavonols, coumarins [19,20] and proanthocyanidins [21].

Phenolic acids belong to a large group of polyphenolic compounds that are the focus of current research due to their ability to counteract the development of cardiovascular diseases, cancers and degenerative disorders, which develop as a consequence of ageing
Previously, authors have reported the presence of protocatechuic, gallic [20], ferulic, chlorogenic [22], neochlorogenic and caffeic acid [11,20-22] in P. spinosa extracts. Flavonoids, particularly anthocyanins, are another large group of secondary metabolites identified in P. spinosa extracts [11,22,23]. Beside their importance for plants, anthocyanins also have significant beneficial effects on human health, since high anthocyanin intakes are associated with decreased risk of various human diseases [24,25]. Proanthocyanidins, which are recognized health-promoting substances due to their antimicrobial, antioxidative, antiinflammatory, antiallergic, vasodilatory and enzyme-inhibitory effects, are also found in blackthorns [20,26]. It is worth mentioning that B-type proanthocyanidins are more widespread in nature, while the A-type is distributed only in Ericaceae, Hippocastanaceae, Lauraceae and some member of Rosaceae, including Prunus species [20].

In spite of their wide usage as food and in folk remedies, blackthorn fruits are still insufficiently explored. Therefore, this study aimed to evaluate the total phenol and flavonoid contents in P. spinosa aqueous, ethanol and acetone fruit extracts. To examine the anthocyanin profile, HPLC analysis was performed and in vitro antioxidant, antimicrobial, antidiabetic and antitumor activities were examined. The correlation between the polyphenol contents and biological properties was considered. To the best of our knowledge, this is the first report of the potential antidiabetic activity of P. spinosa fruit extracts.

MATERIALS AND METHODS

Plant material

P. spinosa fruits were collected from a natural population in July 2015 in Croatia (village Brdine; N 44.5936; E 15.6467) and a voucher specimen was deposited in the Herbarium of the Institute of Botany and the Botanical Garden “Jevremovac” (Voucher No. 17482). Frozen fruits were macerated and extracted with distilled water, 96% ethanol and acetone for 24 h. In order to improve extraction, an ultrasound water bath was used during the first and the last hour. Extracts were then filtered through Whatman filter paper No 1 and evaporated in a rotatory vacuum evaporator (Buchi rotavapor R-114) until dry. Crude extracts were stored at +4°C.

Chemicals and reagents

All chemicals and reagents were at least of analytical grade of purity. The organic solvents used for extraction and experimental procedures (methanol, ethanol and acetone), as well as, sodium hydrogen phosphate dihydrate (NaH₂PO₄·2H₂O), disodium hydrogen phosphate dodecahydrate (Na₂HPO₄·12H₂O), sodium-carbonate anhydrous (Na₂CO₃) and potassium acetate (C₂H₃KO₂) were purchased from VWR Chemicals (Lutherworth, Leicestershire, UK). 2,2-diphenyl-1-picrylhydrazyl (DPPH), the Folin–Ciocalteu phenol reagent, gallic and L-ascorbic acid, 2(3)-t-buty-4-hydroxyanisole (BHA), iron (III) chloride hydrate (FeCl₃·6H₂O), iron (II) sulfate heptahydrate (FeSO₄·7H₂O), potassium hexacyanoferrate (III) [K₃Fe(CN)₆], α-glucosidase type I from baker’s yeast, α-amylase from porcine pancreas, 4-nitrophenyl-α-D-glucopyranoside, Lugol’s solution, [p-iodonitrotetrazolium violet [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), ampicillin were obtained from Sigma-Aldrich, USA. Sodium acetate trihydrate (CH₃COONa·3H₂O) and potassium chloride (KCl) were obtained from Centrohem (Stara Pazova, Serbia) and aluminum nitrate nonahydrate [Al(NO₃)₃·9H₂O] and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) from Fluka Chemie AG (Buchs, Switzerland); [2,2’-azino-bis(3-ethylbenzothiazoline)-6-sulfonic] acid (ABTS) and quercetin hydrate were purchased from TCI Europe N.V. (Zwijndrecht, Belgium), sodium-chloride anhydrous (NaCl) from Superlab (Belgrade, Serbia) and 1% starch solution from Carl Roth Gmbh & Co. KG (Karlsruhe, Germany). Potassium peroxydisulfate (K₂O₈S₂) and trichloroacetic acid (CCl₃COOH) were obtained from Thermo Fisher Scientific (New Jersey, US); glacial acetic (CH₃COOH), concentrated hydrochloric acid (HCl) and ketoconazole were from Zorka Pharma (Sabac, Serbia). The 96-well micro titer plates were obtained from Alfamed d.o.o. (Belgrade, Serbia). Tryptic soy broth (TSB) and malt extract broth (MEB) was purchased from Torlak (Belgrade, Serbia). Glucobay was obtained from Bayer d.o.o. (Leverkusen, Germany).

Evaluation of the total phenol content (TPC)

The TPC was quantified using the Folin–Ciocalteu reagent (FC) [27]. One mL of 10% FC reagent was added
to 0.2 mL of aliquots of the sample and incubated for 6 min. The mixture was supplemented with 0.8 mL of 7.5% sodium carbonate (Na₂CO₃). After 2 h of incubation, the absorbance was measured at 740 nm against the blank (distilled water instead of sample). Results were calculated from the gallic acid (GA) calibration curve (y = 7.0632x − 0.0159) and expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

**Evaluation of total flavonoid content (TFC)**

The TFC was estimated according to the previously reported method [28]. A mixture of 4.1 mL 80% ethanol (C₂H₅OH), 0.1 mL Al(NO₃)₃, and 0.1 mL 1M potassium acetate (CH₃COOK) was added to 1 mL aliquots of sample or ethanol (blank). The absorbance was read after 40 min at 415 nm. The results were obtained from the quercetin (QE) calibration curve and expressed as mg quercetin equivalents (QE) per g of DW.

**HPLC analysis of anthocyanins**

Before injection into HPLC, samples were prepared by dissolving dry extracts in 2N HCl solution in methanol to obtain 5 mg/mL and incubated in a water bath at 90ºC for 1 h. Samples were then centrifuged for 15 min at 6100 x g and the supernatant was evaporated until dry in a rotatory vacuum evaporator (100 mbar, 40ºC). Samples were dissolved in methanol, filtered through NY filter 0.4 µm and injected into HPLC. The HPLC analyses of anthocyanins were performed using the Thermo HPLC UltiMate 3000 and UV-DAD (UV-Diode Array Detector). The column was an AcclaimTM PolarAdvantage II C18 (L=150mm, r=4.6mm, 3µm). The column was kept at 30ºC. Anthocyanins were detected using DAD (200-600 nm). For each sample, 15-30 µL of extracts at concentrations of 10-100 mg/mL was injected in triplicate. The mobile phase was a gradient of three solvents: ddH₂O (A), methanol (B) and 1% formic acid in acetonitrile (C), with a constant flow rate of 1mL/min using the following program: isocratic 0-5 min (A:B:C=90:0:10), gradient 5-20 min (final ratio A:B:C=:=90:0:10), isocratic 20-25 min (A:B:C=:=90:10), followed by a sharp return to the initial conditions and an isocratic 10 min washout (A:B:C=:=90:0:10). Six-point calibration curves were made using anthocyanin standards (delphinidin, cyanidin, malvidin, pelargonidin). Peak detection at 525 nm was used for construction of the calibration curve and quantification of anthocyanins in the samples. Anthocyanin compounds in samples were identified by comparing the retention time of unknown peaks with the reference standards. The total amount of anthocyanins in each sample was calculated.

**In vitro evaluation of the antioxidant activity**

**DPPH assay**

Free radical scavenging activity was evaluated using the 2,2-diphenyl-1-picrylhydrazil (DPPH) method [29] with minor modifications. Briefly, 0.4 mL with different concentrations of the sample (10 to 100 µg/mL) was added to 3.6 mL of a stable DPPH methanol solution and incubated for 30 min in the dark at room temperature. The blank contained methanol instead of the sample. The efficacy of the sample to scavenge DPPH free radicals was calculated from the following equation (Eq. 1):

\[
\text{Percentage of inhibition (\%) } = \left( \frac{A_c - A_s}{A_c} \right) \times 100\%
\]  

(Eq. 1)

where A_c and A_s are the absorbance of the blank and sample solution, respectively. The results were expressed as EC₅₀ values (µg/mL) and compared with known antioxidant compounds, 2(3)-t-butyl-4-hydroxyanisole (BHA) and L-ascorbic acid.

**ABTS assay**

The ability of the extract to neutralize 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) free radicals was measured as described [30]. An ABTS stock solution was prepared 12 h before the experiment by dissolving in 2.46 mM potassium peroxydisulfate (K₂O₈S₂). One mL of stock solution was diluted with 100-110 mL of distilled water to adjust the absorbance value to 0.7±0.2. The final volume of 100 µL of different sample concentrations or distilled water (blank) was added to 4 mL of ABTS working solution. After 30 min of incubation at 30ºC in the water bath, the absorbance was read at 734 nm. Similarly to DPPH, the results were presented as EC₅₀ values, which present the concentration of the extract that causes a decrease
of free radicals for 50%. BHA and L-ascorbic acid were used as standards.

**Ferric reducing antioxidant power (FRAP) assay**

The FRAP assay of extracts was tested as described [31]. The method is based on the ability of antioxidants to reduce iron in complex with 2,4,6-tripyridyl-s-triazine (TPTZ) from the ferric (Fe$^{3+}$) to the ferrous (Fe$^{2+}$) form, which leads to the development of an intense blue color and can be observed spectrophotometrically at 595 nm. Samples dissolved in distilled water were mixed with freshly prepared FRAP reagent (0.3 M acetate buffer solution: 10 mM TPTZ solution in 40 mM HCl : 20 mM FeCl$_3$ = 10:1:1 (v/v/v)) in a final volume of 3 mL and incubated for 10 min at 37ºC. The results were read from the calibration curve constructed using aqueous series dilutions of iron (II) sulfate heptahydrate (FeSO$_4$·7H$_2$O) and expressed as μmol Fe$^{2+}$ equivalents per mg of DW. BHA and L-ascorbic acid were used as a positive control.

**Total reducing capacity (TRC) assay**

The TRC of extracts was estimated according to the reported method [32]. Briefly, different concentrations of aqueous sample solutions (0.2 mL) were mixed with 0.5 mL of 0.2 M phosphate buffer (pH=6.6) and 0.5 mL 1% hexacyanoferrate (III) ([K$_3$Fe(CN)$_6$]). After 20 min incubation at 50°C, the mixture was supplemented with 0.5 mL of 10% trichloroacetic acid (CCl$_3$COOH) and vigorously shaken. The mixture was diluted with 1.7 mL of distilled water and filled with 0.1% iron (III) chloride (FeCl$_3$). Absorbance was read at 700 nm after 30 min incubation at room temperature against the blank which consisted of all reagents except the sample. The results were presented as EC$_{50}$ values calculated from the graph of the absorbance at 700 nm against extract concentration and compared with BHA and L-ascorbic acid.

**Evaluation of antibacterial activity**

The antibacterial properties of the extracts were evaluated by a modified microdilution method [33, 34]. The following Gram-positive bacteria were used: *Bacillus cereus* (clinical isolate), *Micrococcus flavus* ATCC 10240, *Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* NCTC 7973; and the following Gram-negative bacteria: *Enterobacter cloacae* ATCC 35030, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 35210.

The aqueous and ethanol P. spinosa fruit extracts were dissolved in 30% ethanol and mixed in a final volume of 100 µL with TSB in 96-well microtiter plates with a flat bottom. Ten µL of the bacterial inoculum, previously adjusted with sterile saline to a concentration of 1×10$^8$ CFU/mL, were added to obtain the appropriate concentration of the mixture. After a 24-h incubation at 37°C, the lowest concentration causing visible inhibition of bacterial growth (under a binocular microscope) was defined as the minimal inhibitory concentration (MIC). The bacterial strains were stained by the addition of aliquots (40 µL) of 2 mg/mL p-iodonitrotetrazolium violet [2-(4-iodophenyl)-3-(4-nitrophenoxy)-5-phenyltetrazolium chloride] (INT) stain and 30 min incubation. Minimal bactericidal concentrations (MBCs) were determined by serial re-inoculation with 10 µL of the inoculum in 100 µL of liquid TSB in microplates and incubation for another 24 h. The lowest concentration without visible bacterial growth was defined as the MBC, indicating 99.5% killing of the original inoculum. Thirty % ethanol was used as a negative control, and ampicillin was used as a positive control.

**Evaluation of antifungal activity**

Antifungal activity of samples was evaluated against eight micromycetes: *Aspergillus fumigates* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate) by described procedure [34,35]. Fungal spores were washed from agar plate surfaces with 80% (v/v) Tween 80 in 0.85% sterile saline solution and adjusted to a concentration of 1×10$^5$ CFU/mL in spore suspension and stored at 4°C until use. The extracts were dissolved in 30% ethanol and mixed with malt extract broth (MEB) in a final volume of 100 µL in 96-well microplates to make serial dilutions. Then the fungal inoculum was added. After 72 h of incubation at 28°C, MICs were determined as the low-
est concentration without visible fungal growth under a binocular microscope. MBCs were determined by serial re-inoculation of 2 µL inoculums in 100 µL of sterile broth and incubation for 72 h at 28ºC, and defined as the lowest concentrations without visible fungal growth indicating 99.5% killing of the original inoculums. The negative control was 30% ethanol, while the positive control was the commercially available medicine, ketoconazole, an antifungal medication.

**α-amylase inhibitory activity**

The α-amylase inhibitory activity was tested by the Caraway-Somogyi iodine/potassium method as previously described [36] with slight modifications. Different concentrations of sample solution (25 µL) were pre-incubated with 0.5 mg/mL α-amylase solution in phosphate buffer (pH 6.8 with 6mM sodium chloride (NaCl)) (50 µL) for 15 min at 37ºC in a 96-well microplate. The reaction was initiated with the addition of 0.2% starch solution in phosphate buffer (pH 6.8 with 6mM sodium chloride (NaCl)) (50 µL) and incubation for 20 min at 37ºC. Aliquots (25 µL) of 1 M hydrochloric acid (HCl) were used to stop the reaction. Subsequently, iodine-potassium iodide solution (IKI reagent) was added (100 µL) and the absorbance was read at 630 nm. The percentage of the inhibited enzyme was obtained using the following equation (Eq. 2):

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

(Eq. 2)

where \(\Delta A_c\) represents the difference between the control solution (containing all reaction reagents except extract) without and with enzyme solution. Similarly, \(\Delta A_s\) is the difference between the sample solutions without and with α-amylase. The obtained results were expressed as IC_{50} (mg/mL) values and compared to glucobay, an antidiabetic drug used to treat diabetes mellitus type II.

**α-glucosidase inhibitory activity**

The α-glucosidase inhibitory activity was estimated as described [37]. Different concentrations of the sample in final volume of 120 µL were pre-incubated with 20 µL of 0.6U/mL α-glucosidase solution in 0.1 M phosphate buffer (pH 6.8) in 96-well microtiter plates for 15 min at 37ºC. Twenty µL of 3.5 mM \(p\)-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) were added to start the reaction. After 20 min of incubation at 37ºC, the reaction was stopped by the addition of 0.2 M sodium carbonate (Na₂CO₃) and the absorbance was measured at 405 nm. The percentages of the inhibited enzyme were calculated by the following formula (Eq. 3):

\[
\text{Percentage inhibition} \% \times 100\% = \left( \frac{\Delta A_c - \Delta A_s}{\Delta A_c} \right) \times 100\%
\]  

(Eq. 3)

Where \(\Delta A_c\) and \(\Delta A_s\) are the absorbance for the control (containing all reaction reagents except extract) and sample, respectively, subtracted by the control/sample solution without enzyme. The results were presented as IC_{50} values. Glucobay served as the positive control.

**Statistical analysis**

All experimental measurements were carried out in triplicate and the results are presented as their average value (AV) ± standard deviation (SD). Correlations between specific groups of secondary metabolites and antioxidant activities were expressed through Pearson's coefficient of correlation and interpreted according to Taylor [40]. All calculations were performed using MS Office Excel 2007.

**RESULTS**

**Yields, total phenol and flavonoid contents**

Solvents of different polarity were used for extraction and the obtained yields ranged from 9.4% to 18.45% (Table 1). The highest yield was found for the water extract, and the lowest for acetone. The results presented in Table 1 show that the examined samples were scarce in phenol and flavonoid compounds. The TPC ranged from 19.98 to 26.78 mg GAE/g DW being the highest in the acetone extract. The acetone extract had the poorest content of flavonoids (2.89 mg QE/g DW), while the ethanol extract had the highest (3.07 QE/g DW).
HPLC analysis of anthocyanins

The results of HPLC analysis of anthocyanins are presented in Table 1. The sum of the anthocyanins ranged from 9 to 23 µg/g of DW, the highest being in the acetone sample. Four anthocyanin compounds were identified in the ethanol and acetone extracts, delphinidin (Dp), cyanidin (Cy), malvidin (Mv), and pelargonidin (Pg), while in the aqueous extract Pg was absent. Samples were the most rich in Cy among the present anthocyanin compounds.

Table 1. Yields, total phenol (TPC), flavonoid (TFC), delphinidin (Dp), cyanidin (Cy), malvidin (Mv) and pelargonidin (Pg) contents and the sum of anthocyanins (TAC) in P. spinosa fruit extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>TPC mg GAE/g</th>
<th>TFC mg QE/g</th>
<th>Dp µg/g</th>
<th>Cy µg/g</th>
<th>Mv µg/g</th>
<th>Pg µg/g</th>
<th>TAC µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>18.45</td>
<td>23.19±2.52</td>
<td>2.96±0.22</td>
<td>3.00</td>
<td>9.00</td>
<td>2.00</td>
<td>0</td>
<td>14.00</td>
</tr>
<tr>
<td>ethanol</td>
<td>11.09</td>
<td>19.98±1.28</td>
<td>3.07±0.27</td>
<td>1.00</td>
<td>6.00</td>
<td>1.00</td>
<td>1.00</td>
<td>9.00</td>
</tr>
<tr>
<td>acetone</td>
<td>9.4</td>
<td>26.78±4.44</td>
<td>2.89±0.36</td>
<td>4.00</td>
<td>17.00</td>
<td>1.00</td>
<td>1.00</td>
<td>23.00</td>
</tr>
</tbody>
</table>

1 (%)
2 mg GAE/g of dry extract
3 mg QE/g of dry extract
4,5 µg/g of dry extract

Antioxidant activity of P. spinosa fruit extracts

P. spinosa fruit extracts were evaluated in 4 in vitro antioxidant tests and the results are presented in Table 2. The ethanol extract was the most effective in scavenging DPPH, as well as ABTS free radicals, with EC50 values of 257.84 and 184.43 µg/mL, respectively. In both tests, the acetone extract showed the lowest free radical scavenging activity. Similarly, the ethanol extract possessed the best ferric reducing ability, with 0.10 µmol Fe+2 equivalents/g DW in contrast to the aqueous sample. The aqueous sample exhibited the highest antioxidant ability in the TRC assay (EC50=3848.00 µg/mL) while acetone had the lowest antioxidant potential. However, the extracts were a few times less active than antioxidant compounds, BHA and L-ascorbic acid in all used tests.

Correlations between phenols, flavonoids and anthocyanins were expressed through Pearson’s coefficient of correlation (Table 3). A strong negative correlation between phenols and flavonoids was observed, as well as between flavonoids and anthocyanins, which was unusual. A high correlation was established between the phenol content and the values obtained for DPPH, ABTS and TRC tests. In contrast to phenols, flavonoids strongly negatively correlated with the antioxidant activity evaluated by the same assays. Moreover, the correlation of anthocyanin compounds to DPPH and ABTS was similar to that of phenol and to TRC as flavonoid compounds. A moderate negative/positive correlation was observed in the FRAP assay and phenols/flavonoids, respectively. Anthocyanins weakly contributed to the antioxidant activity measured by the FRAP assay. High values of Pearson’s coefficient of correlation were obtained for the relation of DPPH with ABTS and TRC, as well as for the relation of ABTS and TRC. The lowest correlation was found
among FRAP and ABTS/TRC assays with the \( r \) values of 0.1225 and 0.1077, respectively.

### Antimicrobial activity of *P. spinosa* fruit extracts

Antibacterial and antifungal activities of the aqueous and ethanol *P. spinosa* fruit extracts are presented in Tables 4 and 5, respectively. MIC values for antibacterial activity ranged from 5.68 to 11.36 mg/mL and MBC from 11.36 to 22.73 mg/mL. The ethanol extract was more effective against the examined bacteria. *E. cloacae* was the most affected by both samples. Antifungal activity was evaluated against 8 fungal strains and MIC values ranged from 2.87 to 22.94 mg/mL and MFC from 5.73 to 45.87 mg/mL. According to the obtained results, the aqueous extract possessed slightly better antifungal properties. The most susceptible strain to both extracts was *T. viride* (MIC 2.87 mg/mL; MBC 5.73 mg/mL), while *A. ochraceus* was the most resistant to the ethanolic extract. Antibacterial as well as antifungal properties of the examined samples were lower than the commercially used antibiotic and antifungal (ampicillin and ketoconazole, respectively), which served as positive controls.

#### The enzyme-inhibitory activity of *P. spinosa* fruit extracts

Potential antidiabetic activities of the aqueous and ethanol extracts of *P. spinosa* fruits were measured through their ability to inhibit carbohydrate hydrolyzing enzymes, and the results are shown in Table 6. Both samples were more potent inhibitors of \( \alpha \)-glucosidase than of \( \alpha \)-amylase. \( IC_{50} \) values for \( \alpha \)-AIA were 389.83 and 13.44 mg/mL for the aqueous and ethanol extracts, respectively, while for \( \alpha \)-GIA they were 0.22 and 0.08 mg/mL, respectively. Glucobay contains acarbose as an active compound, which has the ability to inhibit \( \alpha \)-glucosidase and \( \alpha \)-amylase and was therefore used as a positive control. From the presented results it can be seen that the ethanol extract displayed significantly stronger inhibition of \( \alpha \)-glucosidase than glucobay.

#### Antitumor activity of *P. spinosa* fruit extracts

Potential antitumor properties of *P. spinosa* aqueous and the ethanol fruit extract on human carcinoma cell
lines (HeLa, K562 and MDA-MB-453) were evaluated by the MTT assay and the results are presented as Supplementary Material. The obtained results with IC\textsubscript{50} values higher than 2000 µg/mL indicate that neither aqueous nor ethanol extracts exhibited antitumor activities on the tested malignant cell lines.

**DISCUSSION**

Solvent polarity, besides temperature, pressure, sonication time etc., has an influence on the group of compounds that are extracted [41]. Thus, in this study, water, ethanol and acetone were used because of their ability to extract different secondary metabolites that could have a role in bioactive responses. Not only is the bioactivity of secondary metabolites important, but also the amounts that are extracted by a specific solvent. Therefore, extraction yields were calculated. Stanković et al. [42] previously examined the extraction yields of *P. spinosa* fruit methanol, 70% ethanol, 45% propylene glycol and water extracts and the obtained results ranged from 19.51 to 31.82% of fresh plant material. The yields reported in this survey were higher, which could be ascribed to differences in the solvent polarity used for the extraction. The value obtained for the yield of the aqueous sample (19.51%) was close to those presented in this research (18.45%).

The total phenol and flavonoid contents of *P. spinosa* fruit extract was previously reported by several authors [11,20,42-45]. Barros et al. [43] examined the phytochemicals in strawberry-tree, blackthorn and dog-rose fruits. Among the examined species, *P. spinosa* fruits possessed the lowest amounts of phenols (83.40 mg GAE/g DW) and flavonoids (8.68 mg CE/g DW), but about 4-fold higher than our samples. Significantly higher values for TPC and TFC in water and ethanol blackthorn fruit extracts were reported by Pinacho et al. [20], which could be explained by differences between the procedures used for extraction. Additionally, the authors used air-dried fruits for extract preparation. However, other authors reported similar amounts of phenols [44,45]. Furthermore, the total flavonoid content in *P. spinosa* fruit extract was previously reported by Stanković et al. [42] was close to the values presented herein. HPLC-DAD analysis of anthocyanins and comparison of obtained peaks with reference compounds enabled the identification of four basic anthocyanins: delphinidin, cyanidin, malvidin and pelargonidin in ethanol and acetone extracts, while in the water extract pelargonidin was absent. The sum of anthocyanins was highest in the acetone extract. To the best of our knowledge, this is the first report of anthocyanins in blackthorn fruit acetone extract. Fraternale et al. [46] previously identified cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside chloride and peonidin-3-O-rutinoside chloride in blackthorn fruits from Italy. A similar anthocyanin profile was determined by Veličković et al. [11]. Popović et al. [45] indicated differences in polyphenols among fifteen wild blackthorn genotypes and aside from the previously mentioned anthocyanins they found hydroxycinnamic acids (3-caffeoyl-quinic acid, 3-p-coumaroyl-quinic acid, 5-caffeoylquvic acid), flavonoids (quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rutinoside, quercetin) and anthocyanin, peonidin-3-rutinoside.

The examined samples of *P. spinosa* possessed antioxidant activity, which is in accordance with literature data [11,20,42-45]. The results presented in this study indicate that among the examined samples, the ethanol extract exhibited the most promising antioxidant properties in three out of the four assays used. The ethanol extract was also the richest in flavonoids, which strongly correlated with the antioxidant properties. Thus, the highest antioxidant activity of the ethanol extract could be ascribed to this group of secondary metabolites. The contribution of flavonoid compounds to antioxidant properties was proven before [11,20,44,47]. Furthermore, anthocyanins strongly correlated with the antioxidant ability estimated by the TRC assay, which is in compliance with previously reported results [11]. Therefore, it could be assumed that anthocyanins had a great contribution to the antioxidant properties of *P. spinosa* fruits as well.

Water and ethanol samples were chosen for investigation of antimicrobial activity due to their non-toxicity. The ethanol extract was more active against the examined bacterial strains, which could also be ascribed to the higher amount of flavonoids in this sample. Several authors previously reported antibacterial activity in blackthorn extracts [11,48,49]. Kumarasamy et al. [48] examined the influence of *P. spinosa* and *P. padus* methanol and dichloromethane seed extracts on seventeen pathogenic bacteria. In the mentioned study, the methanol extract of *P. spinosa*
was active against *L. plantarum* 6376, *S. aureus* 10788 and *Citrobacter freundii* 9750, while the dichloromethane extract showed weak activity against all tested strains. Radovanović et al. [49] examined the antibacterial effects of wild blackberry, European cornel and blackthorn fruit extracts. Their results showed a higher antibacterial activity of the blackthorn extract than of the other two. Among the examined strains, the most sensitive Gram bacteria was *S. enteridis* and the Gram*+* bacteria *S. aureus*. They also noted that MIC values were equal to MBC for some strains, which means that the critical concentration of compounds possessed bactericidal effects. The *P. spinosa* ethanol fruit extract was also tested against *S. abony* NCTC6017, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027 and *B. subtilis* ATCC 6633 [11]. With the exception of *B. subtilis*, all examined bacteria were sensitive to the blackthorn fruit extract, which was linked with the identified flavonoid compounds in the extract. Available literature data about antifungal properties of blackthorn extracts are deficient and contradictory. The influence of *P. spinosa* fruit extract on *C. albicans* ATCC 10231 was noted by Veličković et al. [11], while Gegiu et al. [50] reported a lack of activity. Furthermore, the mold *A. niger* ATCC16404 was also resistant to blackthorn extracts in tests performed by Veličković et al. [11].

The potential antidiabetic properties of blackthorn fruits were evaluated through their ability to inhibit carbohydrate hydrolyzing enzymes. The obtained results demonstrate high enzyme inhibitory potential of blackthorn extracts, particularly on *α*-glucosidase. The obtained results are congruent with those reported by Popović et al. [45] who previously explored *α*-amylase inhibitory activity (α-AIA) and *α*-glucosidase inhibitory activity (α-GIA) of different *P. spinosa* genotypes. Additionally, all examined samples possessed higher α-GIA than acarbose, which is in accordance with our results.

In summary, the examined extracts are a rich source of polyphenols that could be linked with their antioxidant properties. Beside antioxidant activity, the aqueous and ethanol extract displayed antimicrobial and potential antidiabetic activity, as the ethanol extract was particularly active in inhibiting *α*-glucosidase, a carbohydrate hydrolyzing enzyme. Taking into account the presented results, it could be concluded that the ethanol extract of blackthorn fruits is a promising candidate for *in vivo* research of natural compounds with an antidiabetic effect.

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**Author contributions:** IV and SG designed the study. IV, ŽŽ, NR, MI designed, performed the experiments and analyzed the results. IV drafted the manuscript. SG and MS supervised the experiments. SG, MS and PM critically reviewed and edited manuscript. All authors read and approved the final manuscript.

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**REFERENCES**

11. Veličković JM, Kostić DA, Stojanović GS, Mitić SS, Mitić MN, Randelović SS, Đorđević AS. Phenolic composition,


Fang J. Classification of fruits based on anthocyanin types and relevance to their health effects. Nutrition. 2015;31:1301-6.


### Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Velickovic%20et%20al_4932_Supplementary%20Material.pdf