

CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF THE PLUM SEED EXTRACT

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The aim of this paper was to estimate the biological activity of the plum seed extract and to define the chemical composition by using the ESI-MS method. During the investigation of the antioxidant activity, the extract showed a better ability to inhibit DPPH radicals compared with amygdalin standard. The results of the antimicrobial study indicate that the extract has a greater effect on Gram-negative bacteria compared with amygdalin. Gram-positive bacteria and fungi remained resistant in both cases. Unlike amygdalin, the extract showed a greater antimicrobial activity against the same microbiological strains. Amygdalin was isolated from the extract before the investigation of antiproliferative activity against MDA-MB-361, MDA-MB-453, HeLa and LS174 cell lines using MTT assay. Unlike the MDA-MB-453 cell line, the activity of standard and isolated amygdalin was not observed at the concentrations lower than 400 $\mu\text{g cm}^{-3}$ on MDA-MB-361 cell line. The isolated amygdalin has not shown the activity in other cell lines. The antiproliferative activity of amygdalin standard was also noticed on HeLa and LS-174 cell lines at the concentrations of 333.27 and 333.17 $\mu\text{g cm}^{-3}$, respectively.

Keywords: plum seeds; amygdalin; antioxidant activity; antimicrobial activity; antiproliferative activity.

Introduction

Despite a great progress in diagnosis and treatment of malignant tumors, this disease is still one of the causes of death in humans [1]. Today, for the treatment of this disease, the numerous chemotherapeutic preparations can be found on the market. Due to their toxicity, most of the chemotherapy drugs cannot be applied at the doses that can completely inhibit the growth of tumor cells in the organism. Lately, the investigations have been focused on the application of herbal agents, including amygdalin, in the treatment of these diseases. Amygdalin is a natural chemotherapy agent which can be found in more than 1,200 plants, particularly in the seeds of fruits such as apricots, peaches, plums and apples [2]. Actually, it is cyanogen glycoside that contains two glucose molecules in its structure, benzaldehyde (an analgesic) and the cyanide group [3]. Pharmacological studies of amygdalin were mainly related to isolated amygdalin, mostly from apricot (*Prunus armeniaca*) seeds [4]. In pre-clinical studies, Hwang *et al.* [5] proved that amygdalin from *Prunus armeniaca* has an anti-inflammatory activity. In addition, the antibacterial activity of amygdalin isolated from *Prunus armeniaca* was also confirmed [6]. In traditional Chinese medicine, it is known that the seed of *Persicae*, which contains amygdalin as a major component, can be used in treating the symptoms of atherosclerosis [7].

Having this in mind, Jiagang *et al.* [8] confirmed the therapeutic potential of amygdalin in preventing and/or treating atherosclerosis. Recently, Khallouki *et al.* [9] investigated the phytochemical composition and antioxidant capacity of various botanical parts of Mirabelle plums from the territories of France, Germany and Luxembourg. Using the HPLC-ESI-MS method, they defined that the content of amygdalin was over 90% of the phenolic compounds present in the seeds. Using DPPH, FRAP and ORAC assays, it has been observed that amygdalin from these plant materials did not show any antioxidant activity. It is known that amygdalin is highly efficient in "catching" hydroxyl radicals. This characteristic allows the application of amygdalin as a supplement against anti-aging of the human body [10]. In the late 70's and early 80's of the 20th century, it was confirmed that amygdalin shows the antitumor activity in addition to the above mentioned effects. It is believed that amygdalin is a major non-toxic agent for the metabolic treatment of cancer. Although the application of amygdalin has limitations, this bioactive compound has shown to be suitable against tumors of the lungs, breast, prostate, colon and lymphomas [11-13]. Jiagang *et al.* [8] activated amygdalin using β -D-glucosidase enzyme and monitored its effect on Hep G2 proliferative cells. Actually, amygdalin

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has prevented the growth of these cells more strongly after its activation. The extract of *Persicae semen*, which contains amygdalin as a major bioactive compound, was tested on the proliferative cells of leukemia (HL-60). It was found that amygdalin had the cytotoxic effect on HL-60 with IC_{50} of 6.4 mg cm^{-3} in the presence of 250 nM β -D-glucosidase [14].

The data about the investigation of the pharmacological activity of the plum seed extract, and amygdalin isolated from this plant material cannot be found in literature. Due to these reasons, the aim of this paper was to investigate the antioxidant and antimicrobial activity of the plum seed extract, as well as the antiproliferative activity of isolated amygdalin. The qualitative identification of bioactive compounds in the plum seed extract was performed using ESI-MS method.

Experimental

Reagents

Amygdalin standard purity of 97% (Sigma-Aldrich, Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH), a powder medium RPMI 1640, dimethylsulfoxide (DMSO), sodium dodecyl sulfate (SDS), 3-[4,5-dimethyl(thiazol-2yl)-3,5-diphenyl] tetrazolium bromide (MTT), L-glutamine, streptomycin, penicillin, gentamicin (Sigma Chemical Company, St. Louis, USA), fetal bovine serum (FBS) (Gibco BRL, New York, USA), butylated hydroxytoluene-BHT (Centrohema, Stara Pazova, Serbia), absolute ethanol (Alkaloid AD, Skopje, Republic of Macedonia), 96% (v/v) ethanol (Zorka Pharma, Sabac, Serbia), diethyl ether ($\geq 99\%$) (VWR International, Lutterworth, UK).

Plant material

Fresh plum seeds (*Pruni domesticae semen*) was purchased from PD Plemic-commerce (Osecina, Serbia) and dried in a dark place up to the 6% moisture content. The plant material was ground in an electric mill in order to obtain a fine powder with an average particle size of 0.3 mm.

Extraction procedure

The plant material was transferred to the round flask of 100 cm^3 and absolute ethanol was added in the solid-to-liquid ratio of 1:25 (m/v). The extraction was performed under reflux at the temperature of $34.4 \text{ }^\circ\text{C}$. The system temperature was kept constant using a water bath. These operating conditions were applied according to the previously performed optimization study for amygdalin extraction from this plant material using artificial neural network [15]. During the optimization study, the used neural network was a multilayer perceptron MLP (4-3-1) with the exponential function in the hidden layer and the linear function in the output layer. The extract was separated from the solid matrix by filtration on a Buchner funnel. The liquid extract was evaporated in order to remove the residual ethanol on a vacuum rotary

evaporator at $50 \text{ }^\circ\text{C}$. The extract was kept in a desiccator until complete drying, *i.e.* to a constant mass.

ESI-MS/MS analysis

In order to define the qualitative composition of the plum seed extract under optimal conditions, the electrospray ionization coupled with the mass spectrometry method was applied for this analysis. The mass spectra were done on Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer (San Jose, California, USA). The performances of this device enable the identification of the components in the extract without their previous HPLC separation. The presence of bioactive compounds was confirmed based on literature data for mass patterns of the analyzed compounds [9,16]. The sample was ionized using an electrospray (ESI) technique. After ionization, the molecular ions of desired compounds were extracted in the analyzer and treated with the adequate portions of collision energy in order to obtain their MS2 spectra. Mass spectra were recorded depending on the analyte nature in positive and negative ionization modes. The used operating conditions were: ion source voltage of 4.95 kV , ion current of $2.61 \text{ } \mu\text{A}$, the capillary temperature of $275 \text{ }^\circ\text{C}$, and voltage of -50 V , the sheath pressure of 137.14 kPa and auxiliary gas pressure of 13.93 kPa . The MS parameters of this method were adjusted using amygdalin standard. Xcalibur software was used for data collection and analysis.

Preparation of the sample for MS analysis

The stock solutions of amygdalin standard and plum seed extract were prepared by dissolving 25 mg of the sample in 25 cm^3 of methanol. After that, the solutions were diluted from the stock solution to the concentration of $3 \text{ } \mu\text{g cm}^{-3}$ with the addition of the formic acid (0.05 cm^3). The prepared solutions were injected into the MS spectrometer.

Determination of antioxidant activity (DPPH assay)

The procedure of DPPH assay is based on the preparation of a stock solution of investigated samples and on its dilution to make a series of solutions. In this study, the initial concentrations of amygdalin standard, plum seed extract, and BHT were 2.5 mg cm^{-3} , 0.8 mg cm^{-3} and 0.25 mg cm^{-3} , respectively. The prepared solutions (2.5 cm^3) were treated with 1 cm^3 of the ethanolic solution of DPPH radicals ($3 \times 10^{-4} \text{ mol dm}^{-3}$). After that, the samples were incubated at room temperature in a dark for 20 min. The ethanolic solution of DPPH radicals (1 cm^3) diluted with 2.5 cm^3 of ethanol was used as a blank solution. The absorbance of the samples was measured at 517 nm against 96% ethanol. The inhibition of DPPH radicals was calculated based on the Equation 1 [17]:

$$\text{Inhibition of DPPH radicals(\%)} = 100 \times \left(1 - \frac{A_s - A_B}{A_C} \right) \dots \dots \dots (1)$$

where: A_s – absorbance of the samples (ethanol solution of the samples treated with DPPH radicals), A_B – absorb-

ance of the blank (ethanol solution of the samples without addition of DPPH radicals), AC – absorbance of the control (diluted ethanol solution of DPPH radicals).

Determination of antimicrobial activity

The strains of microorganisms used in this study were Gram-negative (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027) and Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6539), as well as fungi (*Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404). Antibiotic agar (10 cm³, pH 6.5) (Sigma Aldrich, Steinheim, Germany) was poured into Petri plates for the bacteria growth, *i.e.* Sabouraud dextrose agar (pH 5.6) (Sigma Aldrich, Steinheim, Germany) for the fungi growth. A medium was previously inoculated with 150 µl of the analyzed cells suspension. The suspensions were prepared by diluting the overnight cultures from liquid medium in a 0.9% NaCl solution to the average content of 10⁸ CFU cm⁻³. The cell number was determined using direct microscopic methods. The plates were kept at 4 °C during 1 h in order to solidify. After that, the sterile discs (Schleicher & Schuell, Dassel, Germany) with a diameter of 12.5 mm were soaked with the ethanolic solutions (30 µl) of the plum seed extract and amygdalin standard and placed on the surface of solidified agar plates. Amygdalin standard was prepared in the concentrations of 0.2, 0.8 and 1.6 mg cm⁻³. Ethanol was used as a negative control. The sensitivity of the strains was also tested to gentamicin in order to compare its antimicrobial activity with the activities of other samples. Bacteria (Gram-positive and Gram-negative) were incubated at 37 °C for 18-24 h, *i.e.* fungi were incubated at 25 °C for 24-48 h in the anaerobic conditions. After the incubation, the diameters of inhibition zones were measured in millimeters. The values were expressed as the mean value of three measurements.

MTT assay

The cell lines

HeLa cells (human cervical adenocarcinoma), MDA-MB-361 (estrogen-dependent) and MDA-MB-453 (estrogen-independent) cells (human breast cancer), LS-174 cells (cells of human colon carcinoma) and healthy MRC-5 cells (human lung fibroblasts).

Nutrient medium

RPMI 1640 medium with 10% of the inactivated bovine serum (FBS) at 56 °C, L-glutamine (3 mmol dm⁻³), penicillin (100 IU cm⁻³), streptomycin (100 g cm⁻³) and HEPES (25 mmol dm⁻³) was used for the growth and maintenance of HeLa, LS-174, MRC-5 cell lines. The pH of medium was adjusted to 7.2 by using the bicarbonate solution. The same medium was used for MDA-MB-361 and MDA-MB-453 cell lines, but with the glucose addition to 1.11 g cm⁻³.

Treatment of cell lines

The samples were dissolved in DMSO up to the ini-

tial concentration of 400 µg cm⁻³. After that, the samples were diluted in the nutrient medium to appropriate working concentrations. Microtiter plates with 96 wells (Nunc, Nalgene, Denmark) were used for the determination of the cell survival. The density of HeLa, MDA-MB-361, MDA-MB-453, LS-174, MRC-5 cells was 2000, 7000, 3000, 7000 and 5000 cells per well in 100 µl of media, respectively. Nutrient medium was used as a blank. After 24 h from seeding the cells, five different concentrations of samples (50 µl) were added to the wells with malignant cells and blanks. Fresh nutrient medium (50 µl) was added to control cells and blanks. The final concentrations of the compounds were 400, 200, 100, 50 and 25 µg cm⁻³. The cells were incubated in a humidified atmosphere (95% air, 5% CO₂) at 37 °C for 72 h.

Determination of cells survival

After the incubation, 20 µl of MTT solution (5 mg MTT/cm³ PBS) was added to each well, while 100 µl of 10% SDS was added after 4 h. The absorbance was measured on an ELISA reader at 570 nm. The antiproliferative effect of amygdalin standard and isolated amygdalin in relation to the control culture of malignant cells was monitored in the experiment. The cells survival was calculated according to Equation 2 [18]:

$$S(\%) = \frac{A_t - A_b}{A_c - A_b} \times 100 \dots\dots\dots(2)$$

where: A_t – absorbance of the samples with the treated cells, A_b – absorbance of the blank, and A_c – absorbance of the control.

Actually, IC₅₀ concentration is defined as the substance concentration that inhibits 50% of the cells survival relative to the untreated control.

Results and discussion

The amygdalin yield was found to be 25.30 g per 100 g of the dried extract, which was determined using the HPLC method described by Savic *et al.* [19]. The antioxidant and antimicrobial activities of the plum seed extract was investigated and compared with the activity of amygdalin standard. Before MTT analysis, the plum seed extract was treated with diethyl ether for the purpose of amygdalin isolation. The solvent was proven as suitable for dissolving the fatty compounds of the extract, while amygdalin was precipitated in the flask as an insoluble polar compound. The precipitated amygdalin was separated by decanting from other compounds in the extract. Its structure was confirmed using various instrumental methods such as UV-Vis, FTIR, ESI-MS methods [15]. This isolated compound was dried in a desiccator before further analysis of its cytotoxic effect on the tested tumor cell lines. The antiproliferative activity of isolated amygdalin was compared with available amygdalin standard.


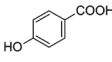
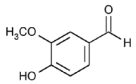
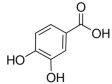
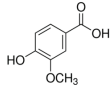
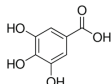
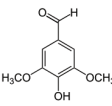
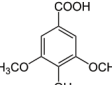
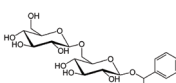
ESI-MS-MS analysis of the plum seed extract

Mass spectrometry method was used for the qualita-

tive analysis of the plum seed extract. The aim of this analysis was to identify the compounds which could act synergistic and affect the antioxidant and antimicrobial activity of the extract. Based on the mass fragmentation patterns described in literature [9, 16], the phenolic and flavonoid compounds were identified in the extract. Khallouki *et al.* [9] also identified the presence of these

compounds in the methanolic extract of plum seeds. The mass fragmentation patterns of the identified compounds are presented in Table 1. The presence of nine bioactive compounds was confirmed in the ethanolic extract of plum seeds. The values of collision energies for each identified compounds are also given in Table 1.

Table 1. The identified bioactive compounds in the plum seed extract using ESI-MS-MS

Bioactive compound	Structure	[M-H] ⁻	ESI-MS-MS fragmentation, m/z	Collision energy, eV
benzoic acid		121.1	106, 93.1, 77*	22
4-hydroxybenzoic acid		136.9	93.1*	19
vanillin		151.1	136.1, 108.1, 92.9*	22
3,4-dihydroxybenzoic acid		153	108.8*	16
vanillic acid		167.3	151.9, 123.2, 107.9, 91.1*	24
gallic acid		169.1	125*	20
syringaldehyde		181	166.2, 151.1, 151.1, 122.7*	23
syringic acid		197.3	182.1, 167, 153.3, 137.8, 122.9, 121, 106.1*	33
amygdalin		[M+Na-H] ⁻ 480	453, 363, 374, 347**	12

*fragmentation patterns described by Khallouki *et al.* [9], and

** fragmentation patterns described by Ge *et al.* [16]

From these results, it can be concluded that a parent ion of benzoic acid gives a signal at m/z 121, while the fragment ions have signals at m/z 93 of $[M-H]^{-}-CO$ and at m/z 77 of $[M-H]^{-}-CO_2$. The p-hydroxybenzoic acid in addition to a parent ion at m/z 137 has a fragment ion at m/z 93, which originates from $[M-H]^{-}-CO_2$. The parent ion of vanillin has a signal at m/z 151. The fragment ions occurred at m/z 136 as a result of the CH_3 group loss, *i.e.* at m/z 108 and m/z 92 originating from $[M-H]^{-}-CH_3-CO$ and $[M-H]^{-}-CH_3-CO-OH$, respectively. The presence of 3,4-dihydroxybenzoic acid was confirmed based on the signal of the parent ion at m/z 153 in the mass spectrum. The effect of the collision energy of 16 eV leads to the appearance of a peak at m/z 108.83 due to a loss of

CO_2 . When vanillic acid with a molecular mass of 168 is recorded in the negative mode, it gives a peak at m/z 167. The action of the collision energy of 24 eV leads to the formation of fragment ions which have signals at m/z 152, 123, 108 and 91. The fragment ion at m/z 152 was caused due to the loss of CH_3 group from the parent ion, while the signal at m/z 123 occurred due to the loss of CO_2 . The ion at m/z 108 was formed after losing CH_3 and CO_2 groups. After further OH group losing, the signal appeared at m/z 91 in the mass spectrum. The gallic acid in its mass spectrum in addition to the parent ion at m/z 169 has a peak of the fragment ion at m/z 125 due to the loss of CO_2 . The loss of CH_3 group from the basic peak of syringaldehyde at m/z 181 causes the signal at m/z

166. After the loss of two CH_3 groups, a new signal at m/z 151 is formed. The ion at m/z 123 occurred after further loss of CO group. Unlike previous two compounds, syringic acid in addition to the parent ion at m/z 197 has a large number of the fragment ions at m/z 182 of $[\text{M}-\text{H}]^- - \text{CH}_3$, m/z 167 of $[\text{M}-\text{H}]^- - 2\text{CH}_3$, m/z 153 of $[\text{M}-\text{H}]^- - \text{CO}_2$, m/z 138 of $[\text{M}-\text{H}]^- - \text{CH}_3 - \text{CO}_2$, m/z 123 of $[\text{M}-\text{H}]^- - 2\text{CH}_3 - \text{CO}_2$, m/z 121 of $[\text{M}-\text{H}]^- - \text{CH}_3 - \text{CO}_2 - \text{H}_2\text{O}$ and m/z 106 of $[\text{M}-\text{H}]^- - 2\text{CH}_3 - \text{CO}_2 - \text{H}_2\text{O}$ [9]. The basic peak of amygdalin at m/z 480 occurred due to the formation of an adduct with sodium $[\text{M}+\text{Na}-\text{H}]^-$. The fragment peaks at m/z 453 and 363 were the result of losing neutral fragments HCN and $\text{C}_8\text{H}_7\text{N}$ [16]. The most common product at m/z 347 and the ionic product at m/z 374 were also noticed because of losing mandelonitrile ($\text{C}_8\text{H}_7\text{NO}$) and $\text{C}_7\text{H}_8\text{N}$ from the parent ion at m/z 480, respectively.

Antioxidant activity of amygdalin and plum seed extract

DPPH assay was applied to investigate the antioxidant activity of amygdalin standard, plum seed extract and synthetic antioxidant BHT. The inhibition of DPPH radicals depending on the sample concentration is shown in Fig. 1. It can be seen that the increase of the amygdalin concentration leads to more effective DPPH radicals inhibition. The maximum value of DPPH radicals inhibition (52.30%) was achieved at the amygdalin concentration of $625 \mu\text{g cm}^{-3}$. Unlike amygdalin that showed a satisfactory antioxidant activity at higher concentrations, the solution of the plum seed extract showed almost the same activity at lower concentrations. The highest inhibition of DPPH radicals of 61.30% was achieved at the concentration of the plum seed extract of $6.3 \mu\text{g cm}^{-3}$. The maximum activity of BHT (80.9%), which also represents the highest value of the investigated samples, was found to be at the concentration of $120 \mu\text{g cm}^{-3}$.

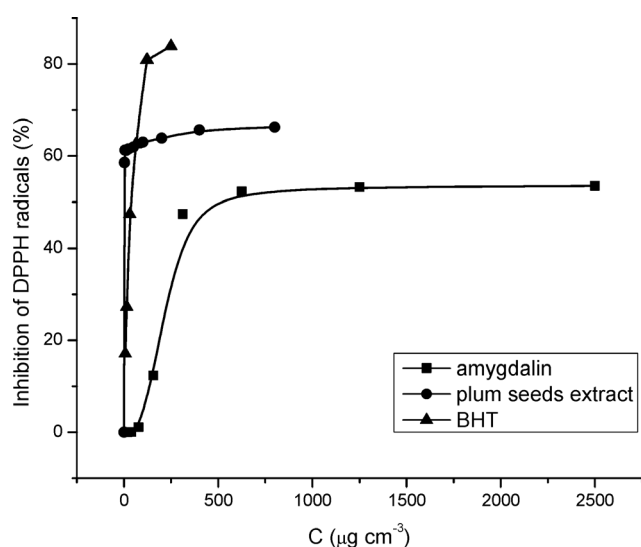


Figure 1. Inhibition of DPPH radicals depending on the concentration of amygdalin standard, plum seed extract and BHT

EC_{50} values of the investigated samples were obtained by an interpolation. This value represents the concentration of the samples necessary to inhibit 50% of the initial concentration of DPPH radicals. The EC_{50} values of amygdalin ($\text{EC}_{50} = 477.8 \mu\text{g cm}^{-3}$) is higher than the value obtained for the plum seed extract ($\text{EC}_{50} = 2.67 \mu\text{g cm}^{-3}$), i.e. than the value obtained for BHT ($\text{EC}_{50} = 36.6 \mu\text{g cm}^{-3}$). Based on these results, it can be concluded that amygdalin has a lower ability to inhibit DPPH radicals compared with the analyzed extract and synthetic antioxidant. A better „scavenge“ability of the extract can be attributed to the presence of additional compounds with an expressed antioxidant activity. This group of compounds usually includes flavonoids and phenolic acids. Khallouki *et al.* [9] investigated the antioxidant activity of the methanol extract of different parts of plum genus *Rosaceae* (*Mirabala*) from France, Germany and Luxembourg. The use of DPPH, FRAP and ORAC assays showed that amygdalin as a major compound of the methanol extract did not have the antioxidative activity. Unlike Khallouki *et al.* [9], the results obtained in our study show that amygdalin and the extract of Stanley plum seeds have a better activity in „scavenging“ of DPPH radicals.

Antimicrobial activity of amygdalin and plum seed extract

Antimicrobial activity of amygdalin standard and plum seed extract was tested on six various strains of the microorganism (four bacteria and two fungi) using a disk diffusion method. The concentrations of the analyzed solutions for amygdalin standard were $C_1 = 0.2 \text{ mg cm}^{-3}$; $C_2 = 0.8 \text{ mg cm}^{-3}$ and $C_3 = 1.6 \text{ mg cm}^{-3}$. The effect of 96% (v/v) ethanol on the microbial growth was also analyzed, because this solvent was used for dissolving the samples. The antimicrobial activity of the samples was compared with the activity of gentamicin. The obtained results are shown in Table 2. The best effect of amygdalin standard was noticed on Gram (-) bacteria, while Gram (+) bacteria and fungi remained resistant to its action. The lowest concentration of amygdalin standard had the smallest effect on Gram (-) bacteria. Among the tested Gram (-) bacteria, *P. aeruginosa* is one of the most sensitive strains on the action of amygdalin standard. The plum seed extract showed the activity only against Gram (-) bacteria. In comparison to the standard, the extract showed a greater antimicrobial activity against the same strains. This is due to the presence of several different classes of compounds (flavonoids, polyphenolic acids and its esters) which probably have the synergistic effects.

In literature, there is a confirmation that the identified compounds in the plum seed extract show the antimicrobial activity. Yurdugül and Bozoglu [20] confirmed that benzoic acid is a major component in a lyophilized sample of wild plum, which is responsible for a good inhibitory activity against the strains of *Klebsiella pneumonia*, *Acinoto iwofii* ATCC 19002, *Enterobacter faecium* ATCC 6057 and *E. coli* O157:H7-933. Benzoic acid is normally present in almost all fruits including cranberries, plums,

raspberries, cloves, cinnamon and other fruits and vegetables [21]. The antimicrobial effect of the phenolic component and p-hydroxybenzoic, vanillin, syringic acid and quercetin has been confirmed in literature [22]. Chanwitheesuk *et al.* [23] have confirmed the effect of gallic acid on some human pathogenic bacteria. It is known that Gram (-) bacteria in the human organism may be a cause of various diseases [24,25] and serious infections [26,27]. *P. aeruginosa* is a part of the physiological microflora of the intestines in humans, and also can be found on the skin. Also, it has the ability to create a great number of toxic proteins which can damage the tissue and cause an immunological insufficiency [28]. Today, antibiotics are used in the treatment of the infections caused by *P. aeruginosa* therapy, because this microorganism is sensitive to cephalosporin antibiotics (cefotaxime, ceftriaxone, cefoperazone) and newer aminoglycoside (ami-

kacin). Pathogenic forms of *E. coli* are responsible for a wide range of human diseases [29]. Actually, *E. coli* is responsible for 90% of urinary tract infections that lead to inflammation of the kidney. Also, it can cause colitis and hemolytic uremic syndrome in humans.

However, in this study it was confirmed that the plum seed extract mainly has the ability to prevent the growth of Gram (-) bacteria (*P. aeruginosa* and *E. coli*). The similar antimicrobial activity of the essential oil of apricot seeds against *P. aeruginosa* and *E. coli* was reported by Lee *et al.* [30]. The obtained results indicate that the plum seed extract can be used for developing new formulations with anti-bacterial or anti-infective effect. A new formulation could be successfully applied for the treatment of infections and other diseases caused by Gram (-) bacteria, such as *P. aeruginosa* and *E. coli* which are resistant to many conventional antibiotics.

Table 2. Antimicrobial activity of amygdalin standard and plum seed extract

Microorganism	Amygdalin standard			Plum seed extract	gentamicin	96% (v/v) ethanol
	C1	C2	C3			
<i>E. coli</i> ATCC 8739	-	++	++	+++	++	-
<i>P. aeruginosa</i> ATCC 9027	++	+++	++++	++++	-	-
<i>B. subtilis</i> ATCC 6633	-	-	-	-	+++	-
<i>S. aureus</i> ATCC 6539	-	-	-	-	++	-
<i>C. albicans</i> ATCC 10231	-	-	-	-	-	-
<i>A. brasiliensis</i> ATCC 16404	-	-	-	-	-	-

It does not show antimicrobial activity (-), inhibition zone <15 mm. A weak antimicrobial activity (+), inhibition zone of 15–16 mm. A moderate antimicrobial activity (++) , inhibition zone of 17–19 mm. A high antimicrobial activity (+++) , inhibition zone of 20–22 mm. A strong antimicrobial activity (++++), inhibition zone >23 mm. The standard deviation ± 0.5 mm.

Anticancer activity of amygdalin and isolated amygdalin from plum seeds

Tumor cell lines (MDA-MB-361, MDA-MB-453, HeLa, LS-174) were treated with different concentrations of amygdalin standard and isolated amygdalin during analyzing the anti-proliferative effect. In addition to these cell lines, the studies were carried out on healthy MRC-5 cells. A colorimetric test based on the application of tetrazolium salt was used for the investigation of the antiproliferative activity. The test generates a signal depending on a degree of cells activation. The results were read on the ELISA reader with a satisfactory precision. The IC_{50} values as a measure of effectiveness of a substance in inhibiting the growth and survival of tumor cell lines are presented in Table 3.

The activity of isolated amygdalin and its standard was not observed at the concentrations lower than $400 \mu\text{g cm}^{-3}$ for the MDA-MB-361 cell line, while for MDA-MB-453 cell lines, the antiproliferative activity was achieved at the concentrations of $304.65 \mu\text{g cm}^{-3}$ (amygdalin standard) and $379.5 \mu\text{g cm}^{-3}$ (isolated amygdalin). The higher IC_{50} value for isolated amygdalin can be ex-

plained based on the fact that the isolate was not completely pure as an available standard. Unlike the isolated amygdalin, amygdalin standard showed the activity towards HeLa and LS-174 cell lines at the concentrations of 333.27 and $333.17 \mu\text{g cm}^{-3}$, respectively. The obtained results for standard and isolated amygdalin are expected, considering that Syrigos *et al.* [31] determined the high IC_{50} value of $40.4 \text{ mmol dm}^{-3}$ for amygdalin on pleomorphic transitional tumor cells (HT1376). This problem was solved with the addition of the β -D-glucosidase enzyme, wherein amygdalin cytotoxicity increased 25 times. All samples did not show the cytotoxicity on the healthy MRC-5 cells. These results are significant because they give priority to the obtained extract and isolated amygdalin for the application as the anticancer agents.

Table 3. IC₅₀ values of amygdalin standard and amygdalin isolated from plum seeds

Compound	IC ₅₀ (µg cm ⁻³)				
	MDA-MB-361	MDA-MB-453	HeLa	LS-174	MRC-5
isolated amygdalin	>400	379.5±10.23	>400	>400	>400
amygdalin	>400	304.65±29.17	333.27±22.35	333.17±71.59	>400

IC₅₀ values are expressed as mean values ± SD determined through MTT assay in three independent experiments.

Conclusion

Amygdalin showed a lower antioxidative activity (52.3%) compared with the plum seed extract (61.3%). Microbiological tests have shown that strains of fungi (*C. albicans* and *A. brasiliensis*) are resistant to the effects of all tested samples. Amygdalin standard and the extract have the antimicrobial activity only to the strains *P. aeruginosa* and *E. coli*. The antiproliferative activity was not observed on MDA-MB-361 cell line unlike MDA-MB-453 cell line at the concentrations of the isolated amygdalin and its standard lower than 400 µg cm⁻³. The isolated amygdalin did not show the anticancer activity on other cell lines. On the other hand, amygdalin standard had the activity on HeLa and LS-174 cell lines at the concentrations of 333.27 and 333.17 µg cm⁻³, respectively. Based on the present results, it can be concluded that the investigated extract has a potential to be used in the pharmaceutical industry for treating various human diseases. A further perspective will be focused on the increase of amygdalin cytotoxicity in the presence of β-D-glucosidase enzyme. The aim will be to add this enzyme in order to increase the hydrolysis of amygdalin and to increase the percentage of the released hydrogen cyanide, which is directly responsible for the antiproliferative activity of amygdalin.

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Izvod

HEMIJSKI SASTAV I BIOLOŠKA AKTIVNOST EKSTRAKTA SEMENA ŠLJIVE

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Cilj ovog rada bila je procena biološke aktivnosti ekstrakta semena šljive i definisanje hemijskog sastava primenom ESI-MS metode. Tokom ispitivanja antioksidativne aktivnosti, ekstrakt je pokazao bolju sposobnost inhibiranja DPPH radikala u poređenju sa standardom amigdalina. Rezultati antimikrobne studije pokazali su da ekstrakt ima veći uticaj na rast Gram-negativnih bakterija u odnosu na amigdalin. Gram-pozitivne bakterije i gljivice ostale su rezistentne u oba slučaja. Za razliku od amigdalina, ekstrakt je pokazao veću antimikrobnu aktivnost na iste sojeve mikroorganizama. Amigdalin je izolovan iz ekstrakta pre ispitivanja anti-proliferativne aktivnosti na MDA-MB-361, MDA-MB-453, HeLa i LS174 ćelijskim linijama upotrebom MTT testa. Za razliku od MDA-MB-453 ćelijskih linija, aktivnost standarda i izolovanog amigdalina nije uočena na MDA-MB-361 ćelijskim linijama pri koncentracijama nižim od 400 $\mu\text{g cm}^{-3}$. Izolovani amigdalin nije pokazao aktivnost na ostalim ćelijskim linijama. Antiproliferativna aktivnost standarda amigdalina uočena je i na HeLa i LS-174 ćelijskim linijama pri koncentracijama 333.27 i 333.17 $\mu\text{g cm}^{-3}$, respektivno.

Ključne reči: seme šljive; amigdalin; antioksidativna aktivnost; antimikrobna aktivnost; antiproliferativna aktivnost.