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Original Scientific Paper

Traditional varieties and wild pear from Serbia: a link among antioxidant, antidiabetic and cytotoxic activities of fruit peel and flesh

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ABSTRACT:
This study was aimed at evaluating the antioxidant and antidiabetic activities of methanolic extracts of peel, flesh and mixed peel, and flesh, as well as the cytotoxic activity of mixed peel and flesh extracts obtained from fruits of six traditional pear varieties (*Vidovača, Lubeničarka, Karamanka, Jeribasma, Lončara* and *Takiša*), one commercial variety (*Williams Bartlett*) and a wild pear (*Pyrus communis*) from Serbia. The tested extracts showed strong antioxidant activity regarding the prevention of β-carotene bleaching and high α-glucosidase inhibition, and no significant cytotoxic potential, with the exception of the *Williams Bartlett* and *Pyrus communis* extracts. Overall, the most potent fruit part was shown to be the peel. The most active variety in all of the applied antioxidant and antidiabetic assays was *Takiša*, while the wild pear, *P. communis*, was the most effective in inhibiting the proliferation of cancer cells. In conclusion, several methanolic extracts of pear fruit are promising candidates for further studies regarding the prevention and treatment of pathological conditions associated with the effects of oxidative stress, such as diabetes and even colorectal cancer.

Keywords:
pear, extracts, antioxidant activity, antidiabetic activity, cytotoxic activity

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INTRODUCTION

The pear (*Pyrus communis* L.) is one of the oldest cultivated plants, being native to Europe and West Asia. This plant is among the most economically important fruit tree crops in temperate zones. Fresh pears are used throughout the world, while they are also commonly used in processed products such as drinks, candies, cakes, dried fruits, jams, etc., making this the ninth most widely produced fruit in the world, cultivated mainly in China, Europe and the United States (Silva *et al.* 2014). In China, a leading world producer of pears, this fruit has been used for nutrition and also as a traditional folk remedy for over 2000 years (Reiland & Slavin 2015).

There are several thousands of pear varieties in the world, however only about 100 varieties are grown commercially. Apart from fruit consumption, various parts of the pear tree have been used in China and Ancient Greece since antiquity (Li *et al.* 2014; Reiland & Slavin 2015). However, this plant still plays an important role in folk medicine in modern times, comprising multiple medicinal uses (Parle & Arzoo 2016). Ethnobotanical studies in the Balkan region have confirmed the use of pears for consumption and medicinal purposes, in treating diabetes, hypertension, high cholesterol, and constipation, as antirheumatic and uroseptic agents, and also for body mass reduction (Jarić *et al.* 2007, 2011; Dajić-Stevanović *et al.* 2014; Savić *et al.* 2019).

The long-time usage of pears in traditional medicine has inspired researchers from different parts of the world to study and verify the medicinal properties of pear parts. The biological activities of the flesh/pulp (mesocarp) and peel (exocarp) or the whole fruits of several pear varieties have already been evaluated. The antidiabetic activity of pears was previously reported by Park *et al.* (2012), Velmurugan & Bhargava (2013), Wang *et al.* (2015) and Wu *et al.* (2015). The meta-analysis of Guo *et al.* (2017) provided evidence of an inverse association between apple and pear consumption and the risk of developing type 2 diabetes (T2D). It has been reported that the results of the antiproliferative and/or cytotoxic effects depend on fruit origin, sample preparation, the cell line used, and many other factors (Sun *et al.* 2002; El-Hawari *et al.* 2018; Živković *et al.* 2018). Furthermore, there are also indications that the daily consumption of pears might reduce the incidence of bladder, lung and oesophageal cancer (Parle & Arzoo 2016).

The human body is continually exposed to different harmful agents, which results in the overproduction of free radicals causing oxidative stress and leading to pathological conditions including T2D, neurodegenerative disorders such as Alzheimer's disease (AD), and cancer. According to various studies, there are indications that T2D increases colorectal cancer incidence by up to three times that of the general population (Yao *et al.* 2014), while the risk of developing AD is increased by 50–60% in the case of T2D (Mittal *et al.* 2016). Nevertheless, there are numerous studies which indicate that plant extracts might be effective in treating both of the aforementioned conditions (Aiello *et al.* 2019; Bar-Shalom *et al.* 2019; Li *et al.* 2019).

This research forms part of a comprehensive investigation of traditional pear varieties in several regions of Serbia, which comprises an ethnobotanical survey and phytochemical analysis of the various bioactivities of fruit peel and flesh. Our previous analyses have shown that chlorogenic acid and arbutin are predominant in peel extracts, followed by quercitrin and isoquercitrin, thus confirming that the peel of traditional pear fruit varieties could be a valuable source of bioactive nutraceuticals possessing health benefits (Savić *et al.* 2021). Moreover, other authors have also reported that peel is rich in polyphenols, which are known to be responsible for several bioactivities (Li *et al.* 2014; Abaci *et al.* 2016).

The aim of the present study was to evaluate and compare the antioxidant, antidiabetic and cytotoxic activities of methanolic extracts of peel, flesh, and mixed peel/flesh obtained from the fruits of six traditional and rare pear varieties (*Vidovača, Lubeničarka, Karamanka, Jeribasma, Lončara* and *Takiša*), one commercial variety (*Williams Bartlett*) and a wild pear (*Pyrus communis* L.) from Serbia.

MATERIAL AND METHODS

Chemicals and reagents. Acarbose, ascorbic acid, disodium hydrogen phosphate dodecahydrate, iron(III) chloride, Lugol's solution, pNPG (4-nitrophenyl β-D-glucopyranoside), potassium dihydrogen phosphate, sodium carbonate anhydrous, sodium chloride, sodium phosphate monobasic dihydrate, α-amylase, α-glucosidase (from *Saccharomyces cerevisiae*) type I, β-carotene 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA). Dipotassium phosphate, disodium hydrogen phosphate dodecahydrate, methanol and sodium phosphate dibasic dihydrate were purchased from VWR, USA. Linoleic acid and Tween 40 were purchased from Acros Organics, Belgium. Furthermore, 1% starch solution was purchased from Carl Roth, Germany. Potassium ferricyanide(III) and trichloroacetic acid were obtained from Superlab, Serbia, while chloroform and hydrochloric acid were purchased from Zorka Pharma, Serbia.

Plant material. For this study the fruits of six traditional pear varieties from organic production were used: *Vidovača, Lubeničarka, Karamanka, Jeribasma, Takiša, Lončara* (from the rural households in central Serbia – Šumadija region); one commercially available variety purchased at a local market, *Williams Bartlett*; and also *Pyrus communis* L., whose fruits were collected from southwest Serbia (N 43°35', E 19°31'). All of the fruits were collected in 2016, at their optimal ripening stage recommended for consumption (from July until October). The collected material was stored at -20°C until the preparation of the extracts.

Preparation of extracts. Before the extracts were prepared, one part of the frozen fruits was peeled to approx. 0.5 mm thickness and afterwards mashed in a jar. This represented the material later analyzed as the peel sample. The remaining flesh (flesh sample) and unpeeled material (mixed peel/flesh sample) were cut into cubes (1 cm^3) and separately homogenized in a stirrer (Waring laboratory blender, No. 8010ES). Afterwards, the pear extracts were prepared using methanol as follows: 10 g of peel was extracted with 10 mL of methanol; 30 g of flesh was extracted with 30 mL of methanol, and the same was also done for the combined peel and flesh. The extractions were performed over 24 hours at room temperature. The extracts were consequently filtered through filter paper (Whatman No.1), evaporated under reduced pressure (Buchi rotavapor R-114) and stored at +4°C until further experiments.

Table 1. The antioxidant activity of the pear methanolic extracts

Pear variety	Extract concentration (mg/mL)	β-carotene bleaching assay (%)			TRP assay (mg AAE/g)		
		Peel	Flesh	Peel + flesh	Peel	Flesh	Peel + flesh
Vidovača	0.5	52.02 ± 0.56^{c}	45.26 ± 0.42^{c}	44.13 ± 0.85^{c}	< 1	< 1	< 1
	1	67.52 ± 0.97^{c}	60.83 ± 0.88^{c}	48.83 ± 1.88^{c}	4.24 ± 0.64	2.76 ± 0.19	20.43 ± 1.16^{x}
	2	76.22 ± 1.39^{c}	68.86 ± 0.88^{c}	69.01 ± 0.41^{c}	15.05 ± 1.12	9.81 ± 0.38	32.90 ± 2.48^{x}
	4	94.69 ± 2.12^{c}	78.83 ± 1.52^{c}	79.81 ± 0.85^{c}	20.24 ± 1.93	13.90 ± 1.09	57.43 ± 2.86^{x}
	6	> 100	85.40 ± 0.42^{c}	83.10 ± 1.41^{c}	32.67 ± 1.48	24.10 ± 1.73	72.14 ± 1.89^{x}
Lubeničarka	0.5	49.47 ± 0.93^{cd}	53.53 ± 1.06^{cd}	35.68 ± 1.83^{cd}	< 1	< 1	< 1
	1	63.27 ± 2.12^{cd}	63.99 ± 1.90^{cd}	47.42 ± 1.64^{cd}	1.00 ± 0.97	< 1	16.33 ± 1.74^{x}
	2	73.67 ± 0.93^{cd}	77.62 ± 0.97^{cd}	61.50 ± 2.35^{cd}	2.52 ± 0.19	4.52 ± 0.27	34.10 ± 0.99^{x}
	4	87.26 ± 1.27^{cd}	82.24 ± 0.24^{cd}	72.54 ± 1.08^{cd}	6.95 ± 0.78	11.52 ± 0.41	61.00 ± 1.86^{x}
	6	91.08 ± 0.37^{cd}	85.64 ± 0.88^{cd}	81.69 ± 1.41^{cd}	11.86 ± 1.00	16.05 ± 0.91	81.67 ± 1.04^{x}
Karamanka	0.5	44.16 ± 2.09^{x,d}	29.77 ± 1.59^{y,d}	37.18 ± 0.98^{xy,d}	< 1	< 1	< 1
	1	59.02 ± 0.93^{x,d}	36.64 ± 0.44^{y,d}	40.17 ± 1.90^{xy,d}	5.33 ± 0.91	37.95 ± 1.49^{x}	4.24 ± 0.64
	2	70.28 ± 0.56^{x,d}	46.56 ± 0.44^{y,d}	52.78 ± 1.71^{xy,d}	9.38 ± 1.91	70.24 ± 1.64^{x}	15.05 ± 1.12
	4	82.38 ± 0.21^{x,d}	57.00 ± 0.67^{y,d}	68.38 ± 0.77^{xy,d}	22.19 ± 0.89	111.19 ± 1.45^{x}	20.24 ± 1.93
	6	87.26 ± 0.97^{x,d}	63.10 ± 0.51^{y,d}	78.21 ± 0.74^{xy,d}	31.29 ± 1.51	114.00 ± 5.20^{x}	32.67 ± 1.48
Jeribasma	0.5	64.89 ± 4.47^{x,ac}	51.34 ± 1.99^{ac}	42.96 ± 1.08^{ac}	1.62 ± 0.34	< 1	< 1
	1	80.66 ± 1.02^{x,ac}	54.50 ± 1.95^{ac}	53.29 ± 1.43^{ac}	20.43 ± 1.16	11.67 ± 0.34	4.00 ± 1.45^{x}
	2	96.18 ± 1.83^{x,ac}	59.61 ± 0.24^{ac}	62.44 ± 1.64^{ac}	32.90 ± 2.48	38.76 ± 0.19	7.67 ± 0.33^{x}
	4	> 100	58.86 ± 0.88^{ac}	72.07 ± 0.85^{ac}	57.43 ± 2.86	64.95 ± 0.85	17.24 ± 0.29^{x}
	6	> 100	76.64 ± 0.42^{ac}	81.22 ± 0.94^{ac}	72.14 ± 1.89	75.57 ± 2.17	23.90 ± 0.67^{x}
Takiša	0.5	81.74 ± 1.39^{x,b}	61.32 ± 2.08^{y,b}	77.86 ± 5.03^{xy,b}	26.29 ± 2.40^{x,a}	4.67 ± 0.66^{a}	19.14 ± 1.41^{a}
	1	95.33 ± 1.85^{x,b}	77.61 ± 1.83^{y,b}	87.10 ± 2.96^{xy,b}	94.81 ± 2.88^{x,a}	8.10 ± 1.05^{a}	19.76 ± 1.75^{a}
	2	> 100	89.82 ± 0.67^{y,b}	93.92 ± 1.35^{xy,b}	128.81 ± 3.37^{x,a}	14.14 ± 0.14^{a}	36.90 ± 1.54^{a}
	4	> 100	96.44 ± 0.67^{y,b}	97.32 ± 1.70^{xy,b}	152.33 ± 5.24^{x,a}	23.52 ± 1.28^{a}	57.43 ± 0.64^{a}
	6	> 100	99.75 ± 0.92^{y,b}	> 100	108.67 ± 5.18^{x,a}	33.00 ± 1.73^{a}	64.05 ± 2.75^{a}
Lončara	0.5	61.78 ± 0.37^{x,ac}	44.78 ± 0.25^{y,ac}	65.69 ± 2.56^{xy,ac}	3.71 ± 1.01^{x}	< 1	< 1
	1	77.07 ± 1.60^{x,ac}	55.22 ± 1.78^{y,ac}	72.26 ± 1.26^{xy,ac}	16.90 ± 5.34^{x}	< 1	5.33 ± 0.91
	2	83.23 ± 0.56^{x,ac}	66.16 ± 1.42^{y,ac}	79.08 ± 3.22^{xy,ac}	34.95 ± 3.19^{x}	< 1	9.38 ± 1.91
	4	92.14 ± 2.15^{x,ac}	86.77 ± 1.42^{y,ac}	87.83 ± 1.48^{xy,ac}	67.81 ± 2.79^{x}	< 1	22.19 ± 0.89
	6	96.60 ± 0.42^{x,ac}	89.31 ± 1.59^{y,ac}	94.65 ± 2.47^{xy,ac}	69.00 ± 5.46^{x}	< 1	31.29 ± 1.51
Williams Bartlett	0.5	65.39 ± 1.11^{x,ac}	58.39 ± 1.93^{ac}	38.50 ± 1.02^{ac}	< 1	< 1	< 1
	1	77.86 ± 1.92^{x,ac}	60.58 ± 5.48^{ac}	51.88 ± 1.02^{ac}	< 1	3.95 ± 0.21	32.52 ± 1.02^{x}
	2	93.64 ± 1.35^{x,ac}	66.18 ± 2.16^{ac}	61.03 ± 0.94^{ac}	< 1	13.62 ± 0.48	61.29 ± 1.44^{x}
	4	> 100	68.37 ± 1.06^{ac}	70.42 ± 0.41^{ac}	2.38 ± 0.21	39.62 ± 0.70	105.29 ± 2.16^{x}
	6	> 100	69.59 ± 2.40^{ac}	71.36 ± 0.47^{ac}	4.71 ± 0.38	62.38 ± 0.83	135.05 ± 1.02^{x}
Pyrus communis	0.5	69.21 ± 0.21^{x,ab}	68.96 ± 1.42^{ab}	50.00 ± 0.41^{ab}	< 1	< 1	0.43 ± 0.22
	1	83.01 ± 0.85^{x,ab}	70.48 ± 0.67^{ab}	65.73 ± 2.48^{ab}	19.76 ± 1.75^{x}	< 1	1.95 ± 0.29
	2	92.36 ± 1.10^{x,ab}	72.52 ± 1.17^{ab}	80.75 ± 0.85^{ab}	36.90 ± 1.54^{x}	< 1	2.52 ± 0.19
	4	> 100	90.84 ± 0.76^{ab}	87.09 ± 0.47^{ab}	57.43 ± 0.64^{x}	< 1	6.95 ± 0.78
	6	> 100	91.86 ± 0.25^{ab}	94.37 ± 1.22^{ab}	64.52 ± 2.68^{x}	6.81 ± 1.08	11.86 ± 1.00
Ascorbic acid	0.5		45.29 ± 0.90^{acd}			-	
	1		64.36 ± 0.68^{acd}			-	
	2		70.00 ± 0.00^{acd}			-	
	4		75.38 ± 0.44^{acd}			-	
	6		86.41 ± 1.12^{acd}			-	

The values are presented as means ± SD (n = 3). For each assay, the mean values with different superscript letters within rows (x-z) and columns (a-d) differ significantly (one-way ANOVA, Tukey's post hoc; $p<0.05$).

Table 2. The antidiabetic activity of the pear methanolic extracts

Pear variety	Extract concentration (mg/mL)	α-amylase inhibitory activity (%)			α-glucosidase inhibitory activity (%)		
		Peel	Flesh	Peel + flesh	Peel	Flesh	Peel + flesh
Vidovača	0.5	< 1	< 1	< 1	< 1	< 1	< 1
	1	< 1	< 1	< 1	< 1	< 1	< 1
	2	< 1	< 1	< 1	< 1	< 1	< 1
	4	< 1	< 1	< 1	83.06 ± 0.61	60.42 ± 1.04	52.14 ± 1.81
	6	1.89 ± 0.19^{x}	< 1	< 1	99.75 ± 0.10	99.83 ± 0.05	96.06 ± 0.01
Lubeničarka	0.5	< 1	< 1	< 1	< 1	< 1	< 1
	1	< 1	< 1	< 1	< 1	< 1	< 1
	2	< 1	< 1	< 1	2.51 ± 0.79	< 1	< 1
	4	2.17 ± 0.32^{x}	< 1	< 1	81.61 ± 1.83	45.43 ± 0.77	86.44 ± 1.07
	6	3.99 ± 0.11^{x}	< 1	< 1	99.89 ± 0.01	>100	97.89 ± 0.19
Karamanka	0.5	< 1	< 1	< 1	< 1	5.65 ± 0.87	< 1
	1	< 1	< 1	< 1	19.53 ± 0.59	9.37 ± 0.48	< 1
	2	< 1	< 1	< 1	52.07 ± 1.54	13.94 ± 0.44	16.52 ± 0.03
	4	< 1	< 1	< 1	98.39 ± 0.61	97.63 ± 0.95	59.17 ± 0.05
	6	< 1	< 1	< 1	99.93 ± 0.03	>100	97.10 ± 0.08
Jeribasma	0.5	< 1	< 1	< 1	14.27 ± 0.82	< 1	< 1
	1	< 1	< 1	< 1	36.30 ± 2.36	< 1	< 1
	2	2.39 ± 0.04^{x}	< 1	< 1	77.67 ± 1.82	< 1	9.91 ± 1.26
	4	5.05 ± 0.27^{x}	< 1	< 1	87.14 ± 0.23	67.68 ± 2.80	94.65 ± 2.32
	6	7.53 ± 0.52^{x}	< 1	< 1	99.85 ± 0.08	>100	98.30 ± 0.05
Takiša	0.5	< 1	< 1	< 1	99.64 ± 0.09^{a}	88.82 ± 0.86^{a}	76.50 ± 0.63^{a}
	1	< 1	< 1	< 1	99.76 ± 0.09^{a}	97.97 ± 0.13^{a}	95.61 ± 0.67^{a}
	2	1.15 ± 0.07^{x,a}	< 1	< 1	> 100	99.93 ± 0.06^{a}	99.70 ± 0.13^{a}
	4	4.94 ± 0.27^{x,a}	< 1	1.54 ± 0.20^{a}	> 100	> 100	> 100
	6	18.49 ± 0.99^{x,a}	< 1	< 1	> 100	> 100	> 100
Lončara	0.5	< 1	< 1	< 1	37.95 ± 1.32^{a}	47.14 ± 1.87^{a}	33.07 ± 2.51^{a}
	1	< 1	< 1	< 1	79.49 ± 1.84^{a}	66.92 ± 0.72^{a}	75.91 ± 1.60^{a}
	2	2.34 ± 0.12^{x}	< 1	< 1	98.48 ± 0.10^{a}	95.24 ± 0.42^{a}	99.69 ± 0.24^{a}
	4	2.72 ± 0.51^{x}	< 1	< 1	99.80 ± 0.16^{a}	99.76 ± 0.05^{a}	99.94 ± 0.11^{a}
	6	1.31 ± 0.05^{x}	< 1	< 1	99.91 ± 0.16^{a}	99.81 ± 0.07^{a}	99.85 ± 0.05^{a}
Williams Bartlett	0.5	< 1	< 1	< 1	< 1	< 1	< 1
	1	< 1	< 1	< 1	3.19 ± 0.71	< 1	< 1
	2	< 1	< 1	< 1	14.84 ± 0.62	< 1	< 1
	4	< 1	< 1	< 1	81.58 ± 2.59	54.57 ± 0.36	63.47 ± 1.81
	6	1.20 ± 0.03^{x}	< 1	< 1	99.77 ± 0.11	99.85 ± 0.17	>100
Pyrus communis	0.5	< 1	< 1	< 1	13.59 ± 0.86	< 1	< 1
	1	< 1	< 1	< 1	63.36 ± 0.44	< 1	< 1
	2	< 1	< 1	< 1	79.37 ± 1.88	< 1	32.39 ± 0.86
	4	1.46 ± 0.05^{x}	< 1	< 1	98.56 ± 0.66	55.43 ± 3.31	95.75 ± 0.96
	6	3.01 ± 0.22^{x}	< 1	< 1	99.87 ± 0.08	97.34 ± 0.12	96.95 ± 0.03
Acarbose	0.5	79.75 ± 1.86^{b}			70.16 ± 1.60^{a}		
	1	90.74 ± 0.55^{b}			83.87 ± 0.48^{a}		
	2	92.64 ± 1.55^{b}			90.35 ± 0.27^{a}		
	4	94.17 ± 0.50^{b}			93.90 ± 0.31^{a}		
	6	95.70 ± 1.49^{b}			94.49 ± 0.12^{a}		

The values are presented as means ± SD (n = 3). For each assay, the mean values with different superscript letters within the same row (x-z) and column (a-d) differ significantly (one-way ANOVA, Tukey's post hoc; $p<0.05$).

Determination of antioxidant activity. For testing the antioxidant activity of the pear extracts two different assays were used, the β-carotene bleaching assay which can detect the ability of extracts to protect β-carotene from bleaching, and a total reducing power assay, based on electron transfer between the substrate and the tested sample.

β-carotene bleaching assay. The β-carotene bleaching assay is based on the discoloration of the orange-yellow color of the β-carotene emulsion, which can be delayed by the addition of an appropriate antioxidant (Ueno *et al*. 2014). The β-carotene bleaching assay was performed according to a slightly modified procedure previously described by Dapkevicius *et al*. (1998). The emulsion was prepared by adding linoleic acid (6.25 μL) and Tween 40 (50 mg) to a solution of β-carotene in chloroform (125 μL, 4 mg/mL), followed by the addition of a further 125 μL of chloroform to the prepared emulsion. The chloroform was then removed using a rotary evaporator (Buchi rotavapor R-114) at 40°C, after which 25 mL of distilled water was added with vigorous shaking. The sample solutions (tested at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2, 4 and 6 mg/mL) and the positive control (ascorbic acid) were prepared in appropriate solvents. Afterwards, 200 μL of emulsion and 28 μL of the test substance (extracts/positive control/absolute methanol as negative control) were mixed. The absorbance was measured prior to incubation (t_0 = 0 min), as well as after 2 h of incubation (t_{120} = 120 min) at 490 nm, using the Multiskan Sky Thermo Scientific microtiter plate reader (Finland). The antioxidant activity of the samples was evaluated in terms of the inhibition of β-carotene bleaching using the following equation:

$$\text{Inhibition (\%)} = (A_{120} - C_{120}) / (C_0 - C_{120}) \times 100, \quad (1)$$

where A_{120} and C_{120} represent the absorbance measured after 120 minutes for the samples and positive controls, respectively, while C_0 symbolizes the absorbance of the negative control measured immediately after the addition of all of the reaction components. All measurements were carried out in triplicate and the results are expressed as mean ± standard error.

Total reducing power. This assay is based on the transformation of Fe^{3+} to Fe^{2+} in the presence of a reductant, which can be detected using a spectrophotometer due to the colorimetric reaction (Jamuna *et al*. 2010). The ability of the extracts to reduce iron (III) was assessed by the slightly modified method of Oyaizu (1986) while following the procedure proposed by Tusevski *et al.* (2014). Briefly, 20 μL of each extract (tested at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2, 4 and 6 mg/mL) was mixed with 40 μL of phosphate buffer (0.2 M, pH 6.6) and 40 μL of 1% potassium ferricyanide (III) solution. The mixture was incubated for 20 min at 45°C, followed by the addition of 40 μL of trichloroacetic acid (10%, w/v), 40 μL of distilled water and 8 μL of 0.1% iron(III) chloride. After 10 minutes incubation at room temperature, the absorbance was measured at 700 nm, using the Multiskan Sky Thermo Scientific microtiter plate reader (Finland). The negative control was prepared in the same manner as the reaction mixture, with the addition of 20 μL of absolute methanol instead of the sample. The total reducing power (TRP) of the samples is expressed as μmol of ascorbic acid equivalents (AAE) per gram of dry extract (μmol AAE/g dry extract). All of the measurements were carried out in triplicate and the results are expressed as mean ± standard error.

Determination of antidiabetic activity. The samples were tested for antidiabetic activity through the inhibition of α-amylase and α-glucosidase activities.

α-amylase inhibition assay. The determination of α-amylase inhibition activity was performed using the slightly modified Caraway-Somogyi iodine/potassium iodide method and according to the methodology previously described by Zengin *et al*. (2014). In brief, 25 μL of properly diluted extracts (tested at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2, 4 and 6 mg/mL) were mixed with 50 μL of 0.5 mg/mL α-amylase enzyme solution prepared in a sodium phosphate buffer (0.1 M, pH 6.8 with 6 mM sodium chloride). After 10 minutes of incubation at 37°C, 50 μL of 0.2% starch dissolved in a phosphate buffer was added and the incubation continued for another 10 min at 37°C. After that, 25 μL of 1 M hydrochloric acid was added to terminate the reaction and 100 μL of Lugol's solution was added for the visualization of the reaction. The absorbance was measured at 630 nm, using the Multiskan Sky Thermo Scientific microtiter plate reader (Finland). Acarbose was used as the positive control. The measurements were carried out in triplicate and the results are expressed as mean ± standard error. The percentage of inhibition of α-amylase activity was calculated according to the following equation:

$$\text{Inhibition (\%)} = (As - Ac_1) / Ac_2 \times 100, \quad (2)$$

where As represents the absorbance of the reaction mixture with the test samples, Ac_1 is the absorbance of the enzyme control (contained buffer instead of the sample), and Ac_2 is the absorbance of the substrate control (contained buffer instead of the enzyme).

α-glucosidase inhibition assay. The determination of α-glucosidase inhibitory activity was performed according to Wan *et al*. (2013). Briefly, 120 μL of extract (tested at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2, 4 and 6 mg/mL) and 20 μL of 0.5 U/mL enzyme solution in a potassium phosphate buffer (0.1 M, pH 6.8) were added to a microtiter plate and incubated for 5 min at 37°C. Then, 20 μL of 5 mM pNPG was added to the mixture and the incubation continued for another 20 min at 37°C. Finally, the reaction was stopped by the addition of 80 μL of 0.2 M sodium carbonate dissolved in potassium phosphate buffer, and the absorbance was measured at 405 nm, using the Multiskan Sky Thermo Scientific microtiter plate reader

(Finland). Acarbose was used as the positive control. The measurements were carried out in triplicate and the results are expressed as mean ± standard error. The percentage of α-glucosidase activity inhibition was calculated according to the following equation:

$$\text{Inhibition (\%)} = (Ac - As) / Ac \times 100, \quad (3)$$

where Ac stands for the absorbance of the negative control (contained buffer instead of the sample), while As represents the absorbance of the reaction mixture with the test sample.

Cell preparation and culturing. The colorectal cancer cell line HCT-116 (obtained from the American Type Culture Collection) was maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin, in optimum conditions, according to standard protocols (Milutinović *et al*. 2015). After a few passages, at 70-90% of confluence, the cells were seeded for MTT assay.

Determination of cytotoxicity. The cytotoxic effects of different pear varieties were determined by MTT assay (Mosmann 1983), a method based on the color-changing reaction of mitochondrial dehydrogenase in living cells with the addition of MTT. The HCT-116 cells were seeded in a 96-well plate (10000 cells/well) and incubated for 24 h, flowing which the cultivation medium was replaced with 100 μL of medium containing the methanolic extracts of the combined peel/flesh from the pear varieties, tested at different concentrations (1, 10, 50, 100, 250 and 500 μg/mL) for 24 and 72 h. The untreated cells served as the negative control. At the end of the treatment period, MTT (a final concentration of 5 mg/mL in PBS) was added to each well and the plates were incubated at 37°C in 5% CO_2 for 2-4 h. The resulting colored formazan crystals were dissolved in DMSO and the absorbance was measured at 550 nm in a microplate reader. The effect on cell viability was calculated as the ratio of absorbance for the treated samples divided by the absorbance of the control sample, multiplied by 100 to provide the percentage of viable cells.

Statistical analysis. All of the experimental measurements were carried out in triplicate and the results are expressed as the average of three measurements ± standard error. The analysis of variance (one-way ANOVA) and Tukey's post-hoc test were performed using PAST (PAleontological STatistics) v.3.21. software (Hammer *et al*. 2001) in order to test the significance of the differences among the mean values. Differences were considered as statistically significant if the *p*-value was less than 0.05.

RESULTS

Antioxidant activity. The results of the β-carotene bleaching assay (Table 1) showed that the peel extracts of the examined pear varieties had the highest antioxidant ability, however, only the peel extracts of *Pyrus communis*, *Jeribasma* and *Williams Bartlett* were significantly more effective in inhibiting the β-carotene bleaching when compared to the flesh extracts and also to the combined peel/flesh extracts. In other cases, the peel extracts showed significantly different activity from the flesh extracts (*Takiša*, *Lončara*, *Karamanka*) or there was no difference whatsoever compared to the other extracts (*Vidovača*, *Lubeničarka*). For the latter, it remained inconclusive as to whether the peel or flesh showed the best antioxidant activity. In terms of the pear variety, *Takiša* and *Williams Bartlett* showed the strongest inhibition of β-carotene bleaching. The variety with the highest activity overall in this assay was *Takiša*, showing excellent results for all of its extracts (ranging from 61.32% to over 100%). From all the analysed pear varieties, only the resulting antioxidant activity of the *Takiša* extracts was significantly different from ascorbic acid, which was used as the positive control. Nevertheless, the *Karamanka* extracts showed the lowest capacity to inhibit β-carotene bleaching (results ranging from 29.77% to 87.26%).

The TRP assay, on the other hand, painted a different picture of the activity of the extracts (Table 1). Namely, the peels of *Takiša*, *Lončara* and the wild pear were significantly more active than their flesh alone and the peel/flesh combined. For *Karamanka* and *Jeribasma*, the flesh extracts showed higher antioxidant activity, which was significantly different only in the case of *Karamanka*. On the other hand, the extracts of the combined peel/flesh of the *Vidovača*, *Lubaničarka* and *Williams Barrlet* varieties had the highest TRP. The results obtained from the TRP assay showed that only the *Takiša* extracts exhibited significantly different iron reduction potential when compared to the other tested pear varieties (ranging from 26.29 to 152.33 mg AAE/g), while the *Lončara* flesh extract had the lowest values (less than 1 mg AAE/g on all concentrations).

Antidiabetic activity. The results of the antidiabetic activity of the tested extracts, evaluated through the inhibition of α-amylase and α-glucosidase, are presented in Table 2.

Interestingly, none of the extracts inhibited the activity of α-amylase on concentrations lower than 2 mg/mL (Table 2). Moreover, the inhibition of this enzyme was only detected for several peel extracts. The *Takiša* extracts were significantly more active (values ranging from 1.15 to 18.49%) than the other analyzed pear varieties. These results, however, were not notable since the positive control, acarbose, inhibited the activity of the aforementioned enzyme more prominently (from 79.75% on 0.5 mg/mL to 95.70% on 6 mg/mL).

Unlike in the previously discussed assay, the extracts exhibited significant activity in the α-glucosidase assay, achieving almost complete inhibition of the enzyme at the highest applied concentration (Table 2). Although the peel extracts were more effective than the flesh and peel/flesh combined extracts, significant differences regarding

the used fruit parts were not established. All of the tested *Takiša* and *Lončara* extracts showed significantly higher α-glucosidase inhibition activity, which was similar to the results obtained for acarbose. The *Takiša* peel extract exerted the greatest α-glucosidase inhibitory potential, inhibiting 53.84% of the enzyme activity at the concentration of 0.05 mg/mL (data not shown), making this extract the most active when compared to the other extracts, and it inhibited the enzyme even more efficiently than acarbose. The lowest activity, on the other hand, was observed for the *Vidovača* and *Williams Barrlet* extracts.

Cytotoxic activity. The influence of the combined peel/flesh extracts of the pear varieties on HCT-116 cell viability was evaluated using the MTT assay. The cells were treated with different concentrations of the extracts for 24 and 72 h in order to examine the roles of dose and time of exposure. Figure 1 show that some varieties (*Vidovača*, *Lubeničarka*, *Karamanka* and *Williams Barrlet* only after 72 h, and *Pyrus communis*) dose-dependently decreased the cancer cell viability, where a significant inhibition on cell viability was observed in higher applied concentrations of the extracts, while lower concentrations produced no activity, stimulated cell proliferation or generally showed weak effects. No impact on HCT-116 cell viability was found for the treatment with the *Jeribasma* extracts. On the other hand, the *Karamanka* and *Takiša* extracts stimulated the cell proliferation.

Moreover, prolonged treatment (72 h) with the extracts caused significant inhibition of cell viability in the case of each pear variety, with the exception of *Jeribasma* and *Vidovača*. For the treatment with the *Jeribasma* and *Vidovača* extracts it was found that time of exposure had no influence on the cells. Furthermore, it was generally observed that cell viability decreased more rapidly after the 72 h than the 24 h treatment, except for the treatment with the *Vidovača* extract. The wild pear, *P. communis*, was the most effective in inhibiting the proliferation of these cancer cells overall.

DISCUSSION

Pear fruits represent a valuable source of antioxidants (Manzoor *et al*. 2013; Li *et al*. 2014; Abaci *et al*. 2016; Azzini *et al*. 2019), however, an extensive literature survey showed that data on the biological potential of *Vidovača, Lubeničarka, Karamanka, Jeribasma, Lončara* and *Takiša* fruits remains scarce.

In this study, peel, flesh and combined peel/flesh methanolic extracts of different pear varieties, *Vidovača, Lubeničarka, Karamanka, Jeribasma, Lončara, Takiša, Williams Bartlett* and a wild pear (*Pyrus communis*) from Serbia, were investigated for their ability to act as antioxidant agents in two different chemical systems, the β-carotene bleaching assay and the total reducing power assay.

First of all, the obtained results suggest that pear peel extracts have stronger antioxidant activity compared to the flesh extracts, which was also confirmed in earlier studies. Namely, Manzoor *et al.* (2013) showed that methanolic extracts of the peel of two pear varieties from Pakistan inhibit linoleic acid peroxidation more efficiently than their pulp, while also showing a higher reducing power compared with the pulp. Moreover, Li *et al*. (2014) found that the reducing capacity of the peels of Chinese pear cultivars is higher than that of the pulps. These findings are entirely consistent with our results. According to the existing literature data (Manzoor *et al*. 2013; Li *et al*. 2014; Wang *et al*. 2015; Morgado *et al*. 2019; Savić *et al*. 2021.), the difference in activity between peel and flesh extracts might be attributed to the presence of a higher content of compounds such as polyphenols (arbutin, catechin, chlorogenic acid, *p*-coumaric acid, epicatechin, ferulic acid, hyperoside, isoquercitrin, quercitrin, rutin and vanillic acid) and triterpenes (oleanolic acid and ursolic acid) in the pear peel compared to the flesh, since those compounds are mainly responsible for the displayed antioxidant activity.

As previously explained, oxidative stress plays a major role in the pathogenesis of diabetes and its further complications. Since the majority of the tested pear varieties proved to be valuable antioxidant agents, their antidiabetic potential was tested through their inhibitory effect on α-amylase and α-glucosidase activity. α-amylase catalyzes the hydrolysis of internal α-1,4-glucosidic linkages in starch from food products for a short period of time, however, this enzyme is also an important component of the pancreatic juice (Barbosa *et al*. 2013). Furthermore, α-glucosidase catalyzes the final step of intestinal carbohydrate digestion. By inhibiting intestinal α-glucosidase, the digestion and absorption of carbohydrates will be delayed, and subsequently the earliest metabolic abnormality to occur in T2D, postprandial hyperglycemia, will be suppressed (Wang *et al*. 2015). However, due to various side effects related to the high inhibition of these enzymes (hypoglycaemia, liver problems, lactic acidosis), which emerged after using some of the existing drugs, scientists have turned to nutraceuticals as an efficient strategy to control the disease and also to provide safe benefits without the unwanted secondary effects of the currently available drugs (Tundis *et al*. 2010; Barbosa *et al*. 2013).

The results presented in this study showed that the tested pear extracts do not possess the ability to efficiently inhibit the activity of α-amylase, which was not the case with the reference drug, acarbose. Tundis *et al*. (2010) reported that molecules with the ability to form quinones or lactones or substances with a 4-oxo-pyrane structure are prone to induce the inhibition of α-amylase. However, since the methanolic extracts in our study did not inhibit the activity of α-amylase, the fault might lie in the choice of the solvent used for the extraction process, since Barbosa *et al.* (2013) showed that alcoholic extracts exhibited a lower range of inhibitory activity compared to aqueous ones. Moreover, Tundis *et al*. (2010) reported that some substances exhibit significantly reduced reactivity due to

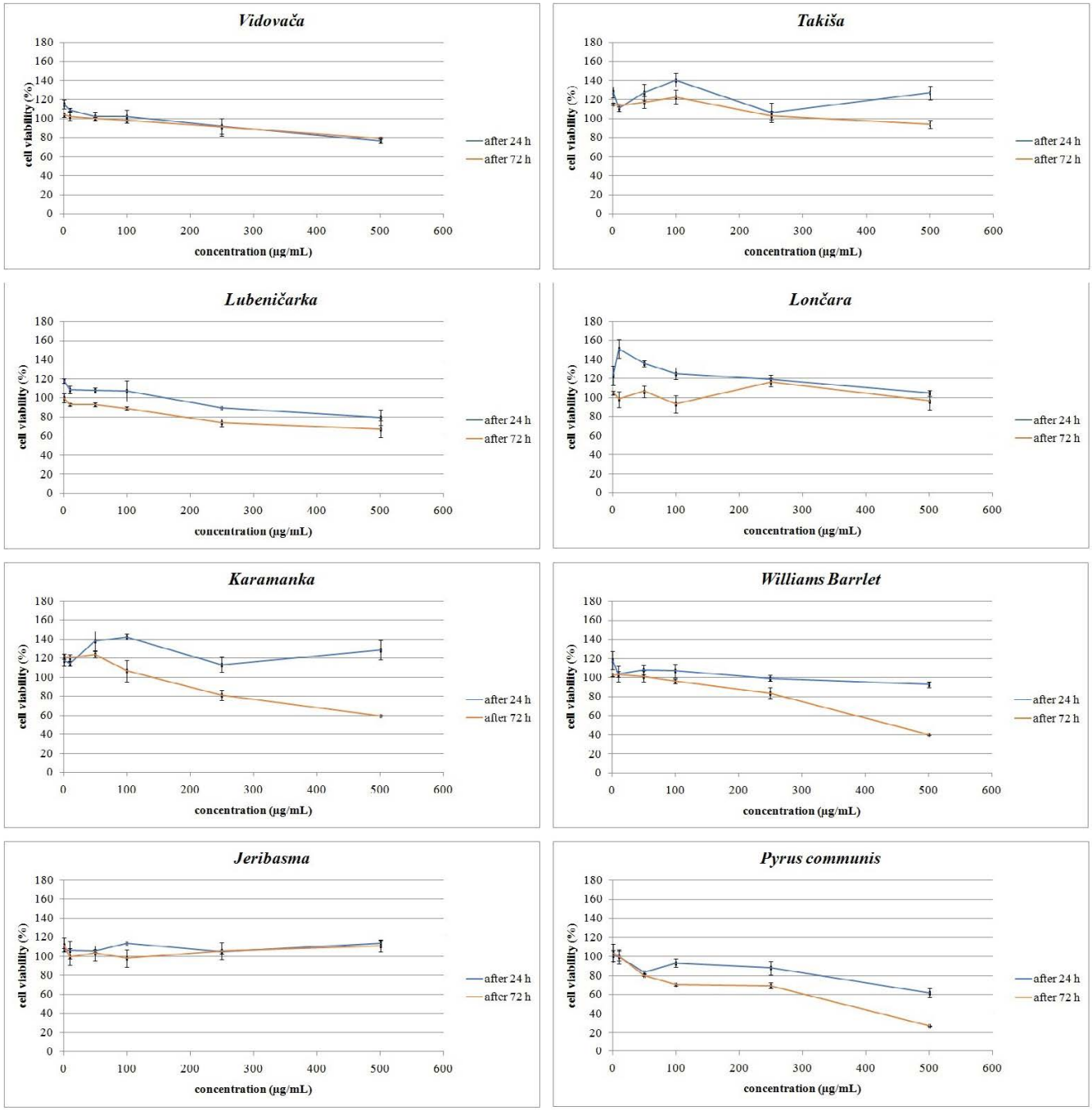

Fig. 1. The cytotoxic activity of the pear methanolic extracts

the existing methoxy groups, steric obstructions or short chain length; hence the culprit for the lack of activity of the pear extracts may also be sought in their chemical composition. Savić *et al.* (2021) suggested that the analyzed pear extracts possess a high phenolic compound content; however, the results presented in this study suggest that those phytochemicals cannot be responsible for the inhibition of α-amylase, which was also previously reported by Barbosa *et al.* (2013).

On the other hand, the tested extracts exhibited inhibitory effects on α-glucosidase with the highest inhibition detected for the *Takiša* peel extract. While investigating six different pear varieties, Barbosa *et al.* (2013) found a possible correlation between the qualitative and quantitative composition of polyphenols, dose and proportional inhibition of α-glucosidase, which is in accordance with our current study and Savić *et al.* (2021). The possible mechanism of action of the presented extracts towards

α-glucosidase activity might be through the formation of hydrogen bonds between the hydroxyl groups of the inhibitory compounds from the extracts and catalytic residues in the active site of α-glucosidase, since the binding of a substance to the active site of the enzyme induces conformational changes in the enzyme, thus causing its loss of function (Lunić *et al*. 2020). Meanwhile, further research concerning the lack of activity observed in the α-amylase assay needs to be carried out.

Since there is a proven link between free radicals, T2D and colorectal carcinoma, this study was designed to analyze the potential of the fruits of different pear varieties to prevent and act against these health disorders. Although the link between the biological mechanism of T2D and colorectal cancer prognosis is still not sufficiently studied or understood, this association might be primarily based on the effects of hyperinsulinemia, insulin resistance and pathogenesis on the insulin/insulin-like growth factor system, whose role is crucial in the pathogenesis, progression and prognosis of colorectal carcinoma (Zhu *et al*. 2017). The final step in this study was to analyze the antiproliferative/cytotoxic activity of the pear extracts using the MTT assay.

The results of the MTT assay showed that some of the investigated varieties have the potential to inhibit the proliferation of colorectal cancer cells, suggesting the need for a further investigation of their anticancer potential on other types of cancer cell lines. Also, a link was observed between the duration of the treatment of the cells with the pear extracts, where all of the tested varieties, except for *Jeribasma*, caused inhibition of cell proliferation after prolonged treatment. An extensive literature survey indicated that there is not enough data on the cytotoxic activity of pear extracts against HCT-116 cells, however, there are studies involving certain phytochemicals from pears with the potential to prevent and treat colorectal cancer. The potential of the dietary bioactive compounds to affect tumorigenesis in all steps including initiation, promotion and progression were previously investigated and reported by Costea *et al*. (2018). Although it can be hypothesized that these extracts might express anticancer activity by modulating the production of reactive oxygen species, since they were shown to exert strong antioxidant activity (Wang & Yi 2008), the latter cannot be proven at this point since this study did not include the testing of this mechanism of action. Considering the availability of pears as a food and the observed mild anticancer potential on colorectal cancer cells, this research should be continued with regard to their combined treatments with more pronounced cytotoxins, while also paying attention to their interactions. It has been reported that the treatment of colorectal carcinoma might be made more efficient through a synergistic approach – by combining naturally occurring substances with certain drugs (Milutinović *et al*. 2015; Costea *et al*. 2018), which is the general goal in healthcare nowadays.

CONCLUSIONS

The current study was aimed at comparing the bioactivity of the methanolic extracts of the peel, flesh and combined peel/flesh parts of six traditional varieties which have not been previously studied, one commercial, and one wild pear in the light of the effects associated with oxidative stress. The tested extracts showed high antioxidant activity regarding the prevention of β-carotene bleaching and high α-glucosidase inhibition. Generally poor to mild antiproliferative activity was observed against the tested colorectal cancer cell line. Finally, the most potent fruit part was shown to be the peel. Furthermore, *Takiša* proved to be the most active variety in all of the applied antioxidant and antidiabetic assays, while the wild pear, *P. communis*, was the most effective in inhibiting the proliferation of cancer cells.

Based on the presented data, it can be concluded that methanolic extracts of pear fruit are promising candidates for further studies regarding the prevention and possible treatment of certain pathological conditions associated with the effects of oxidative stress, such as diabetes and even colorectal cancer. Since the fruit extracts of these pear varieties showed promising results in this study, but the precise mechanisms of action and their detailed chemical composition are not yet known, further *in vitro* and *in vivo* studies should be aimed at comprehensively analyzing their biological potential and developing more effective nutraceuticals for preventing and treating various pathological conditions, free from harmful side-effects.

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REZIME

Botanica SERBICA

Tradicionalne sorte i divlja kruška iz Srbije: veza između antioksidativne, antidijabetične i citotoksične aktivnosti kore i mesa plodova

Mariana Oalđe Pavlović, Ana Alimpić Aradski, Aleksandra Savić, Smiljana Janković, Milena Milutinović, Petar D. Marin i Sonja Duletić-Laušević

U okviru ovog istraživanja urađena je procena antioksidativne i antidijabetične aktivnosti metanolnih ekstrakata kore, mesa i kombinovanih uzoraka kore i mesa, kao i citotoksične aktivnosti kombinivanih ekstrakata kore i mesa dobijenih iz plodova šest tradicionalnih sorti krušaka (Vidovača, Lubeničarka, Karamanka, Jeribasma, Lončara i Takiša), jedne komercijalne sorte (*Williams Bartlett*) i divlje kruške (*Pyrus communis*) iz Srbije. Testirani ekstrakti su pokazali snažnu antioksidativnu aktivnost u β-karoten testu i visoku inhibiciju α-glukozidaze, dok su citotoksični efekat pokazali samo ekstrakti *Williams Bartlett* i *Pyrus communis*. Generalno, kora se pokazala kao najpotentniji deo ploda. Najaktivnija sorta u svim primenjenim antioksidativnim i antidijabetičnim testovima bila je Takiša, dok je divlja kruška, *P. communis*, pokazala najbolji citotoksični efekat. Na osnovu dobijenih rezultata, pojedini metanolni ekstrakti plodova kruškaka su se pokazali kao dobri kandidati za dalje studije prevencije i lečenja patoloških stanja povezanih sa efektima oksidativnog stresa, poput dijabetesa i karcinoma debelog creva.

Ključne reči: kruške, ekstrakti, antioksidativna aktivnost, antidijabetična aktivnost, citotoksična aktivnost