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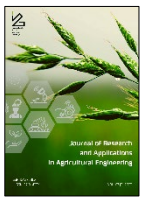

The Effect of Extraction Process on The Antioxidant Activity of *Prunus Serrotina* L. Leaves Extracts

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The study evaluated the antioxidant potential of *Prunus serotina* L. leaves extracts obtained with the use of purified water in the Mowo Aqua system, prepared in 5, 10 and 15 minutes. It was found that the purification system affects the properties of the obtained extracts. First of all, the osmolality of the extracts increased, which indicates the amount of extracted compounds. Water purification in the tested system resulted in an increase in the content of compounds reacting with the Folin Ciocalteu reagent. Their highest content was found in extracts prepared within 10 and 15 minutes. Higher content of polyphenols was responsible for higher antiradical activity in tests with DPPH radicals (5.41mg TE/1g d.m.) and ABTS cation radical (8.69 mg TE/1g d.m.). High antioxidant activity was also confirmed in the FRAP test, where extracts prepared with Mowo Aqua water showed up to 27% higher activity compared to control extracts. The above results were confirmed by the electrochemical method.

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1. Introduction

Plant raw materials are a source of many nutritionally ingredients. Literature confirm the functional and even health-promoting effects of fruits, vegetables and herbs. Recently, the subject of great scientific interest are less known plants or underutilized species that could be used in food technology. Particular attention is paid to invasive species that may have a negative impact on the environment. Such a plant is *Prunus serotina* L, which is common throughout Europe and Asia Minor. Bird cherry (*Prunus* L.) is a type of tree or large shrub belonging to the subfamily *Amygdaloideae* (= *Prunoideae*) within the *Rosaceae* family [1]. This species includes about twenty types of plants, and two of them are the most popular: bird cherry *Prunus padus* L. and bird cherry *P.serotina* This, in turn, is a typical invasive plant that currently dominates the local *P. padus* [2].

Bird cherry is known for its anti-inflammatory, antimicrobial and antioxidant properties. The components of bird cherry show antioxidant activity and may be responsible for reducing inflammation by improving the oxidative-antioxidant balance in the body [3]. Leaves, herbs, fruits, coffee or tea leaves in everyday use are ingredients of beverages, after prior extraction with water. This process is designed not only to produce an aromatic infusion, but also to influence the process in such a way that the content of bioactive compounds and functional properties are the most beneficial. Therefore, an important factor, apart from variable parameters such as extraction time or temperature, are the parameters of the extractant, i.e. water [4].

Concerns about drinking tap water are deeply rooted in the minds of people in Poland. As research shows, consumers are increasingly using water purification systems in their households. Although tap water meets all standards and is constantly monitored by water supply companies and relevant sanitary services, water performance parameters, such as taste, smell and hardness, are not satisfactory everywhere and vary in individual cities, and sometimes also in individual city districts, sometimes raising reservations of users [5]. The purification systems are also equipped with new systems for enriching water with minerals or hydrogenation, which may have a beneficial effect on the antioxidant activity of meals or drinks prepared with it.

The aim of this study was to evaluate the effect of the extraction method of bird cherry *P.serotina* leaves on polyphenolic compounds and the antioxidant activity of the extracts. The paper evaluates the effect of water purification in the tested system on the antioxidant potential of bird cherry leaf extracts measured by spectrophotometric and electrochemical methods.

2. Material and methods

2.1. Material

The leaves of *P.serotina* were harvested in the orchard farm in Ozierany Małe in Podlasie, Poland (53º 13' 14.865" N 23º 51' 9.327" E). Soil in the orchard was characterized by an average abundance of macronutrients. Approximate value of pH for soil, marked in 1M KCl, was 6.12. the content of humus was 1.13%. The leaves were stored in frozen conditions (temperature -28°C) until lyophilization and the extracts prepared. Lyophilization was performed in a CHRIST 1-4 LSC freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) under constant conditions. The condensation temperature in the freeze dryer was maintained at −28 °C, the temperature on the freeze dryer shelf at −20 °C, and the product temperature at −4 °C. The entire process was carried out under reduced pressure for 24 h. The leaves were extracted after grinding in Grindomix GM 200 (Retsch, Haan, Germany) for 180 seconds at 1700 x *g* at 21°C.

2.2. Analytical methods

2.2.1. Preparation of plant extracts

The water extract from *P. serotina* was obtained using water, at 95°C, 1000 mL of extractant was mixed with 50 g of crushed raw material and extracted for 5,10 and 15 minutes. The extractions were carried out with the use of tap water from the municipal intake in Poznań and the same water but purified with the *Mowo Aqua* system. The purification involved the use of 3 types of filters, i.e RO – reverse osmosis filter element (QLS-RO50G, water flow 0,13 L/min, pressure 0,4-0,8 MPa); PAC - folding PP cotton activated carbon rod composite filter (50-150mmx60-510 mm, flow 3L/min, pressure 0,4-0,8 MPa); FC rear composite carbon rod (size 30-150 x 60-510 mm; water flow 1,5 L/min, pressure 0,4-0,8 MPa). The plant material macerated for the specified time without additional heating during the process.The extracts were filtered and centrifuged (800 x *g*, 15 minutes) each time. The fractions were decanted and filtered (Whatman 1:11 μm). The prepared extracts were stored in dark tubes until examination at 4°C.

2.2.2. Color and osmolality of extract measurment

The CIELAB system is used for quality control of color products, where a production sample is compared to the production color standard. Color differences are calculated and compared to the tolerances specified by the customer. These tolerances are usually agreed between the supplier and the customer, taking into account their experience and commercial requirements. The CIELAB system enables effective quality control of color products and helps adjust tolerances to individual customer needs. Color of leaves extract were measured. Color measurement was run in L* a* b* CIE unit system using spectrometer CM-5 (Konica Minolta, Japan) according to methodology described by the device producer. As a source of light, D 65 was applied, and color temperature equaled 6504 K. The observation angle of standard colorimetric observer was 10°. Measurements for each sample was repeated fivefold. The instrument calibration was performed with use of black pattern. Osmolality was measured using an osmometer (Semi-MikroK-7400S)

as the concentration of dissolved substances in a solution, expressed as the number of moles of osmotically active substances (osmoles, Osm) dissolved in one kilogram of solvent (water).

2.2.3. Antioxidative potential analysis by spectrophotometric method

The total phenolic content (TPC) of the obtained extracts was determined using the method described by Kulczyński et al., 2016 with minor modifications [6]. Aliquots of 100 µL diluted in 900 µL of 40% ethanol (Sigma-Aldrich, Germany) were mixed with 1 mL of Folin–Ciocalteu reagent (Sigma-Aldrich, Germany), followed by the addition of 1 mL of 35% sodium carbonate (POCH, Poland). Samples were vortexed for 5 s and after incubation in darkness at room temperature for 90 min, the absorbance of the reaction mixture was measured at 765 nm against blank. The TPC was expressed as mg of gallic acid (Sigma-Aldrich, Germany) equivalents (GAE) per 1 g (mg/1 g) of dry mass using the calibration curves of gallic acid (y=3,65x+0,5).

The DPPH procedure was based on the reduction of DPPH solution absorbance (2,2-diphenyl-1-picrylhydrazyl) at wavelength 517 nm in the presence of free radicals [6]. Measurements were performed using SP-830 Plus apparatus (Metertech, Taiwan). The percentage of DPPH radical scavenging was evaluated on the basis of the standard curve for y = 321.54x + 21.54 (R^2 = 0.986) and presented as Trolox equivalents (mg TE / 1 g d.w of extract).

The ABTS cation radical scavenging activity was measured according to the EAC (Trolox Equivalent Antioxidant Capacity) test according to the methodology described by Kobus-Cisowska et al. 2020 [7]. Spectrophotometric measurement of the ability to scavenge $ABTS^{\bullet+}$ formed from ABTS (2,20-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) by oxidation with potassium persulphate was carried out at a wavelength of 414 nm using SP-830 Plus apparatus (Metertech, Taiwan. The percentage rate of $ABTS^{\bullet+}$ scavenging was calculated from the standard curve for y = 121.63x +26.33 (R^2 = 0.96) and expressed as mg TE/g d.w. of extract.

The antioxidant properties of the extracts were determined using a ferric reducing/antioxidant power assay (FRAP method) according to procedure described by O'Sullivan et al. 2013 [8]. FRAP reagent was added to 1 µL of each sample diluted in 999 µL distilled H_2O. A calibration curve was constructed using $FeSO_4$ $7H_2O$. Samples were incubated for 30 min and the absorbance was measured at 593 nm (Metertech SP880, Taiwan). Data were expressed as µM $FeSO_4$ / 1 g d.w of extract.

2.2.4. Antioxidative potential analysis by electrochemical assay

The content of redox compounds in *P. serotina* leaves extracts was determined using square wave voltammetry (SWV). Voltammetric measurements were performed using potentiostat PGSTAT12 with the GPES 4.9 control software (EcoChemie, The Netherlands). A three-electrode measuring system consisting of a reference electrode Ag/AgCl (3 M KCl) (Mineral, Poland), platinum as an auxiliary electrode (Mineral, Poland) and carbon paste as a working electrode (CPE) was used for the measurements. The CPE was developed according to a described procedure [9]. Carbon paste was made by mixing graphite powder (Sigma) with mineral oil (Sigma) in the ratio of 70:30 (w/w). The surface of the CPE was renewed before use by removing the outer layer of carbon paste on filter paper, application of fresh paste and polishing it to a smooth finish on a frosted glass microscope slide. Before electrochemical measurement, the surface of CPE was treated with 0.05 M phosphate buffer mixed with 0.01 M KCl (pH 7.0) at a potential of +1.7 V for 60 s. After that the electrodes were immersed for 120 s in the solution containing extract dissolved in phosphate buffer in the ratio 1:1 (v/v), whether the SWV measurement in the range from −0.3 V to +1.4 V was made. Applied SWV parameters were: step potential of 5 mV, frequency of 50 Hz and amplitude of 40 mV. Three repetitions of SWV measurement for each extract were performed. SWV voltammograms were smoothed using Savitzky–Golay's method [10]. From SWV voltammograms the baselines determined with moving average procedure was subtracted and finally were determined the data including peak potential, peak height (current), peak area for each signal, and the total peak areas. On the basis of the our results for Cornus mas extracts [12] were also determined an electrochemical index (EI) describing the electrochemical activity of tested extracts, expressed (calculated) as the total area of all redox signals, in relation to 1 g dry matter of examined extract.

Statistical analysis

Statistical analysis of the all results were performed using Microsoft Excel 2013 software (USA) and Statistica 13 software (StatSoft, Poland). All assays were conducted in triplicates and results expressed as mean ± SD. One way ANOVA testing was used to analyze statistical differences. The *p* value less than 0.05 was assumed as a level of significance. The electrochemical results were treated as an additional factor to the model based on standard analytical techniques. The *p* values for Levene's test of independent variables were calculated.

3. Results

3.1. Characteristics of *Prunus serotina* L. extracts

P.serotina extracts were physically and chemically characterized (Table 1). It was shown that the color of the tested extracts differed in terms of assessed parameters. Parameter L* determining the brightness was 33.75±1.19 in the sample with purification system, extracted in 15 minutes. Parameter a*, responsible for the color change in the range from green to red, was 10.51±0.93 for control extraction process in 10 minutes and 12.33±0.12 for extraction with tested system in 5 minutes, whereas parameter b* responsible for the color change in the range from blue to yellow had lower values for control extraction process in 10 minutes (5.54) and higher for extraction in puricication System in 15 minutes - 11.51.

Osmolality ranged from 0.165 to 0.198 and the freezing temperature was the lowest for *P. serotina* leaves extracted in 10 minutes in the tested System (- 0278).

Table 1. Characteristics of the tested *P.serotina* leaves extracts, given in CIE L*a*b* units and osmolality

Sample	Extraction with purification system			Control extraction process		
	5 min	10 min	15 min	5 min	10 min	15 min
Osmolality (mOsm/ kg H_2O)	0.175^{c}±0.04	0.185^{c}±0.02	0.198^{d}±0.03	0.165^{a}±0.02	0.169^{a}±0.03	0.171^{b}±0.04
Freezing temperature (°C)	$-0{,}277^{b}$± 0.01	$-0{,}278^{b}$± 0.01	$-0{,}269^{b}$± 0.01	$-0{,}262^{a}$± 0.01	$-0{,}261^{a}$± 0.01	$-0{,}255^{a}$± 0.01
L*	32.44^{b}±1.01	33.36^{b}±1.21	33.75^{b}±1.19	29.44^{a}±0.84	28.54^{a}±0.73	29.58^{a}±0.16
a*	12.33^{b}±0.12	11.45^{b}±0.43	11.19^{b}±0.55	10.76^{a}±0.65	10.51^{a}±0.93	10.63^{a}±0.41
b*	10.23^{b}±0.22	10.76^{b}±0.84	11.51^{b}±0.73	5.56^{a}±0.98	5.54^{a}±0.52	5.68^{a}±0.57

Results are mean values of three determinations ± standard deviation. Values sharing the same letter in a line are not significantly different ($P \leq 0.05$).

3.2. Antioxidant activity

The obtained extracts were evaluated in terms of antioxidant potential by spectroscopic methods (Table 2). It was found that extracts made from bird cherry (*P.serotina*) leaves using tap water and purified water in the tested system differed in properties. The tested samples contained compounds reacting with the Folin-Ciocalteu reagent. The highest content of these compounds was found for the *P.serotina* leaves extract extracted with water in the purification system for 15 minutes (36.23 mg GAE/g DM), and the lowest for control extracts prepared for 5 minutes (21.54 GAE/g DM). The results of the research were deepened by determining the effect of the extracts using the DPPH radical test. The extract prepared for 15 minutes using water from the tested purification system was shown to scavenge radicals at the highest level of 5.41 mg TE/1g d.m. Tests carried out using the ABTS radical method confirmed that the highest antiradical activity was obtained for extracts obtained from the tested purification system. In the FRAP test, *P. serotina* leaves extracts prepared with purified water showed approximately 25% higher activity compared to tap water-based extracts.

Antioxidant activity is commonly measured by *in vitro* spectrophotometric methods, however, electrochemical techniques are particularly well suited to the analysis of the antioxidant properties of polyphenolic compounds related to their ability to donate electrons. This allows for the selective detection of these compounds with good sensitivity even in very complex systems such as plant extracts. Table 3 summarizes the electrochemical parameters determined for the tested *P.serotina* extracts. Higher electrochemical activity expressed as the total area of redox signals and the electrochemical index (EI) determined on its basis was found in the *P. serotina* leaves extract obtained by water purification. The total signal area was 3.941 V×μA for the 15 minute extract, while the EI was 125.831. Definitely the lowest electroactivity was found in all extracts constituting the control sample.

Table 2. Antioxidant activity of *P.serotina* leaves extracts with FRAP, DPPH, ABTS radicals and total polyphenol content

Sample	Extraction with purification system			Control extraction process		
	5 min	10 min	15 min	5 min	10 min	15 min
Folin-Ciocalteu (mg GAE/g dw)	30.21^{b}±12.17	35.33^{b}±2.32	36.23^{b}±1.43	21.54^{a}±3.34	23.36^{a}±1.22	23.54^{a}±3.31
DPPH (mg TE/1g dw)	4.73^{b}±0.21	5.24^{b}±0.03	5.41^{b}± 0.06	3.43^{a}±0.03	3.59^{a}±0.04	3.63^{a}±0.04
ABTS mg TE/g dw	8.55^{a}±0.08	8.62^{a}±0.04	8.69^{a}±0.06	6.76^{b}±0.11	6.43^{b}±0.02	6.67^{b}±0.03
FRAP μM $FeSO_4$/1g dw	184.31^{b}±3.87	189.73^{b}±2.64	204.32^{b}±5.11	158.85^{a}±2.66	161.98^{a}±2.66	162.41^{a}±1.32

Results are mean values of three determinations ± standard deviation. Values sharing the same letter in a line are not significantly different ($P \leq 0.05$).

Table 3. Electrochemical parameters determined for *P.serotina* leaves extracts

Signal current – Square Wave Voltammetry (SWV)						
Sample *P.Serrotina* bark extract	**Peak potential** E_P [V]	**Peak current** I [μA]	**Peak area** $E_P \times I$ [V × μA]	**Total peak current** I [μA]	**Total peak area** [V × μA]	**EI** [V x μA / 1 g d.m.]
Extract of *Prunus serotina* leaves with purification system						
95°C / 5 minutes	0.227	3.588	0.723	7.432	1.984	63.699
	0.610	3.587	1.121			
95°C / 10 minutes	0.273	7.925	1.519	12.761	3.422	109.226
	0.585	4.843	1.848			
95°C / 15 minutes	0.239	9.624	1.844	15.365	3.941	125.831
	0.554	5.618	2.108			
Control extract of *Prunus serotina* leaves						
95°C / 5 minutes	0.233	4.457	0.905	7.399	1.424	45.162
	0.645	2.933	0.421			
95°C / 10 minutes	0.262	4.136	0.838	9.187	2.742	87.571
	0.559	5.027	1.814			
95°C / 15 minutes	0.255	5.542	1.152	10.879	3.122	99.643
	0.547	5.337	1.963			

4. Discussion

Extraction is a process for the separation of substances by diffusion, by dissolving them in a suitable solvent, and then separating them from the other components of the sample. This process consists in the transition of one or more components from the matrix (solution or solid) to the second phase - the extractant (usually a liquid). Extraction processes at home is importance, because consumers obtain infusions of herbs and teas using water as an extractant [13, 14]. Therefore, the comparison of the impact of water purification on the quality of *P. serotina* leaves extracts in this work is not only of objective importance, but also indicates

the need for further research on the impact of this system on the properties of other infusions as well. In industry, depending on the physico-chemical properties of the sample and the bioactive substances to be extracted, an appropriate solvent or a mixture of solvents is selected [8, 15]. Regardless of whether these are domestic or industrial conditions, the choice of extractant depends mainly on the degree of solubility of the substances in it. It is also important that the efficiency of the extraction process also depends on the properties of the primary matrix itself, such as fragmentation. The *P. serotina* plant and its individual anatomical parts are known for their anti-inflammatory, antimicrobial and antioxidant properties [15]–[19]. The use of a new water purification system and the extraction of bird cherry leaves with it confirms the beneficial properties in terms of antioxidant potential. The literature repeatedly indicates that the content of polyphenols not only in *P.serotina* but also in other raw materials determines the antioxidant properties, the mechanism of action of which can be multidirectional [15]. As it was shown in the work, the quality of the water used for their preparation may affect the properties of the extract. The treatment system that was used in this study subjected tap water to three-stage filtration. Its system uses a cotton filter with a carbon fiber rod, reverse osmosis technology and carbon fiber composite filtration. The device both purifies water and mineralizes it, and additionally enriches it with hydrogen molecules, which are a natural antioxidant. In recent years, interest in natural plant sources has increased in the context of their possible antioxidant and anti-radical activity [3, 12]. The conducted research showed that the leaves of the *P.serotina* plant may have such properties. There are many studies available in the literature on the subject. In the DPPH radical test, the extract prepared for 15 minutes with purified water showed the highest activity and it scavenged radicals to the highest degree. In the DPPH study by Hyun et al. 2015 [17] the highest activity was shown by the extract of 300 mg/mL concetration for leaves 0.88 ± 0.00 μg/mL. In another study, activity in the DPPH test according to Olszewska et al. 2016 [14] for leaves, it was at the level of 2.10-2.29 ug/g. Polyphenols can inhibit the formation of free radicals, can scavenge them and can increase the catalytic activity of endogenous enzymes involved in the neutralization of free radicals. Donno et al. 2018 evaluated the antioxidant potential of individual morphological parts of this plant, and it was shown that the highest antioxidant potential in the FRAP test was found in the bark 379.35b uM FeSO4/1g d.w. [19]. In this study, in the FRAP test, *P.serotina* leaves extracts prepared with water purification system showed about 27% higher activity compared to tap water control extracts. The electrochemical method, square-wave voltammetry, which was used in this work, has a significant advantage over other methods enabling the determination of the level of 8-oxoguanine, because it allows direct testing of the DNA sample without the need to hydrolyse it, which is necessary in other highly sensitive methods [9]. Because to the use of this method, it was confirmed that three-stage water purification makes it possible to obtain extracts with a higher content of polyphenolic compounds and a higher antioxidant potential.

P.serotina leaves are a good source of polyphenolic compounds with high antioxidant activity. The total antioxidant effect is difficult to predict, because the total antioxidant capacity is determined not only by the properties of individual compounds, but above all by their mutual interaction, as well as the quality of the water used to prepare the extracts and the compounds contained in it. The research confirmed the diversity of the mechanisms of action of antioxidants contained in *P.serotina* leaves, as well as the diversity in terms of the results obtained in terms of the water quality used to prepare the extracts. It was confirmed that the three-stage water purification system increases the efficiency of extraction of polyphenolic compounds from bird cherry leaves and thus increases the antioxidant activity. It is necessary to carry out further work in order to confirm how purified water can affect the extraction of active compounds from other plant matrices and their mixtures of dietary and prophylactic importance.

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