Fruit Growing Research, Vol. XXXVIII, 2022 DOI 10.33045/fgr.v38.2022.13
https://publications.icdp.ro/index.php

ACTIVITATEA ANTIOXIDANTĂ ȘI PROFILUL FENOLIC AL FRUCTELOR UNOR VARIETĂȚI ROMÂNEȘTI DE ARBUȘTI

ANTIOXIDANT ACTIVITY AND PHENOLIC PROFILE OF FRUITS FROM SOME ROMANIAN SHRUB FRUIT VARIETIES

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Abstract

The berries are rich in polyphenols, vitamins, and other bioactive compounds, and exhibit antioxidant, antimicrobial, anti-inflammatory and anticancer activities. In this work, antioxidant activity (AA) and a phenolic profile of berries fruits belonging to the *Aronia melanocarpa* (Michx.) Elliott, 'Melrom' cv., *Lonicera caerulea* var. *kamtschatica*, 'Kami' cv., and *Sambucus nigra* L., 'Elrom' cv. were studied. All three cvs. were previously patented by the Research Institute for Fruit Growing Pitesti, Romania. Alcoholic extracts from berries were obtained by four methods: continuous, maceration, ultrasounds and microwaves-assisted. The antioxidant content of extracts was evaluated by an HPLC method. AA was determined by DPPH free radical scavenging method. Total phenol, flavonoid, and anthocyanin contents were determined by spectrophotometric methods. The AA values were reported as equivalents (μg mL^{-1} extract) caffeic and gallic acids, morin, quercetin, rutin, and are in concordance with the results of the chromatographic method. In addition, the chromatographic method allowed identifying the *p*-coumaric acid, caffeic acid, chlorogenic acid, rutin, and epicatechin. The experimental results have shown that analyzed fruit extracts have high antioxidant activity, due mainly to flavonoids and anthocyanins in high concentration. Therefore, extracts from mentioned berries can be used in different nutraceutical products with high antioxidant potential.

Cuvinte cheie: arbuști, activitate antioxidantă, polifenoli, flavone, antociani.
Key words: berries, antioxidant activity, polyphenols, flavonols, anthocyanins.

1. Introduction

It is known that the berries fruits have many important biological activities, such as antioxidant, antimicrobial, anti-inflammatory, anticancer, etc. (Jurikova et al., 2017; Młynarczyk et al., 2018; Paredes-Lopez et al., 2010), due to the content of phenolic acids (chlorogenic acid), polyphenols (flavonoids, anthocyanins), vitamins (vitamin C), and other bioactive compounds. For example, *Aronia melanocarpa* (black chokeberry) is one of the richest sources of polyphenols in the plant kingdom (Denev et al., 2018).

Berries are used in a lot of products: juice, nectars, syrups, wine, tinctures, pastries, jams, dairy products, etc. (Ochmian et al., 2010; Sidor and Gramza-Michałowska, 2019). Chokeberry (which have an astringent taste) and elderberry fruits are rarely consumed fresh (Młynarczyk et al., 2018; Sidor and Gramza-Michałowska, 2019).

Antioxidant activity (AA) and phenolic profile of fruits belonging to the *Aronia melanocarpa* (Michx.) Elliott, 'Melrom' cv (*Rosaceae*, chokeberry), *Lonicera caerulea* var. *kamtschatica*, 'Kami' cv (*Caprifoliaceae*, honeysuckle berries) and *Sambucus nigra* L., 'Elrom' cv. (*Adoxaceae*, elderberry) were studied. These cvs. were introduced in fruit industry by the Research Institute for Fruit Growing Pitesti, Romania (Ancu et al, 2016; Mladin et al., 2013; Titirica et al., 2019) Alcoholic extracts from mentioned berries were obtained by: continuous extraction (Soxhlet), maceration, ultrasonically assisted extraction and microwave-assisted extraction. The antioxidant content of extracts was evaluated by a HPLC method (Ghinea et al., 2021). Antioxidant activity (AA) was determined by DPPH free radical scavenging method (Lungu et al., 2010) and expressed as caffeic acid equivalent (CAE), gallic acid equivalent (GAE), morin equivalent (ME), quercetin equivalent (QE), and rutin equivalent (RE). The total phenol (TP), flavonoids (TF) and anthocyanidins (TA) contents of plant extracts were spectrophotometrically estimated by using chlorogenic and caffeic acids, morin, quercetin, and, respectively, rutin as standards (Lungu et al., 2010, Popa et al., 2012). A comparison between results from this study and some literature was made.

2. Material and methods

Chemicals. Chemicals used in this study are as follows: caffeic acid, chlorogenic acid, morin (2′,3,4′,5,7-pentahydroxyflavone) hydrate, 1,1- diphenyl-2-picrylhydrazyl (DPPH), cyanin chloride (all from Sigma-Aldrich); quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) dihydrate, rutin (3,3′,4′,5,7-pentahydroxyflavone 3-rutinoside, quercetin 3-rutinoside) trihydrate, Folin-Ciocalteu reagent, sodium carbonate (all from Merck); gallic acid monohydrate (Riedel-deHaën); aluminum chloride hexahydrate, potassium acetate (Scharlau, Spain), acetonitril (Supelco), trifluoroacetic acid (TFA) (Sigma-Aldrich), ethanol (S.C. Reagents Com S.R.L., Romania).

Plant materials. The berries belonging to the *Aronia melanocarpa* (Michx.) Elliott, 'Melrom' cv., *Lonicera caerulea* var. *kamtschatica*, 'Kami' cv. and *Sambucus nigra* L., 'Elrom' cv. were studied. All three cvs. were previously patented by the Research Institute for Fruit Growing Pitesti, Romania.

Plant extracts preparation. The extracts from mentioned berries were obtained by: continuous extraction (Soxhlet, SOX), maceration (MAC), ultrasonically assisted extraction (UAE) and microwave-assisted extraction (MAE) (Lungu et al., 2010; Vinatoru et al., 2017). Fresh fruits were soaked and then extracted with 96 % ethanol (vegetable material: solvent ratio = 1:10 w/v). For continuous extraction, it was used a Soxhlet apparatus and the extraction time was 3 h. The same extraction solvent and the same ratio vegetable material/ solvent were used for the maceration at room temperature, five days, with occasional stirring. UAE was carried out using a simple ultrasonic cleaning bath (Brandsonic MH1800 Brandson, 40 kHz), extraction time 2 x 30 minutes, no heating. Alcoholic extracts obtained were filtered and the alcohol was evaporated at room temperature in Petri dishes (9-10 cm) to constant weight. The obtained extracts (semi-solid extracts) were kept at -20 °C.

To obtain the microwave extracts, 5 g of fruit ground in 50 mL of 96% ethanol were introduced into the tubes of the extraction apparatus (Milestone Start D microwave extraction system). The tubes were inserted into the microwave extraction system. The time required for the in fact extraction was 60 minutes at 100 °C. The obtained extracts were cooled to room temperature in tubes, then filtered under vacuum and stored at -20 °C until analysis.

Chromatographic method

Sample preparation. Approximately 10 mg of each extract obtained by Soxhlet extraction, maceration and UAE (according to the methods described above) was dissolved in 1.5 mL solution with 96% ethanol. To promote the dissolution of the extracts, the solutions were introduced into the ultrasonic bath for 10 minutes at room temperature. Microwave extracts were obtained prior to analysis. For this reason, the freshly obtained extracts were analyzed. Immediately prior to determination, all extracts were filtered through a 0.20 µm membrane filter (Teknokroma) and placed in vials.

HPLC Polyphenols determination. To separate and identify the principal compounds with antioxidant activity from extracts, High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was used as the method of analysis (Ghinea et al., 2021). The method was carried out using the HPLC Systems L-3000 from RIGOL TECHNOLOGIES, INC, Beijing, China, with Kinetex EVO C18 (150 × 4.6 mm, particle size of 5 µm) column. The injection volume was 10 µL. The mobile phase consisted of a two-solvent system, used in gradient elution: (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in acetonitrile. The gradient elution was 2−100 % B. The elution flow was set at 1 mL/min, the column temperature was 30 °C and the elution time was 60 min. (Ghinea et al., 2021)

Chromatograms of the analyzed extracts were obtained at wavelengths: 230 nm, 250, 280 and 300 nm. Identification analyses were performed by comparison with standards spectra at each retention time. The chromatograms obtained were processed with the UltraChrome WorkStation RIGOL TECHNOLOGIES program.

Free DPPH radical scavenging activity

Sample preparation. 0.010 g semi-solid extract were dissolved in 96% ethanol, such that 10 mL ethanolic solution from each extract were obtained, except extracts result by MAE, which were analysed as such (because the solvent was not evaporated).

For determination of free radical scavenging activity of extracts, DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was used (Afolayan et al., 2008; Lungu et al., 2010,). This method is based on the reduction of purple DPPH· by an antioxidant, which has as result the yellow coloured DPPH-H (1,1-diphenyl-2-picrylhydrazine) molecule (Lungu et al., 2010). To 500 µL alcoholic extract or standard solutions were added 500 µL DPPH (0.01 g% in ethanol 96%) solution. After 30 min. in dark, at room temperature, absorbance (UV-mini 1240 Shimadzu spectrophotometer) was measured at 536 nm against ethanol in a 1-cm glass test tube. This wavelength value was chosen from the absorbance wavelength spectrum (at the mentioned wavelength the absorbance had the highest value). It was worked with 30 µg mL^{-1} standard solutions. The DPPH radical ability for neutralizing the free radical was expressed as a percentage of inhibition (I%) and was calculated using the formula (1):

$$I(\%) = (A_C - A_S) / A_C \times 100 \quad (1)$$

where A_C = the absorbance of the ethanolic solution of DPPH·;
A_S = the absorbance of sample/standard.

The calibration curves I(%) (percentage of inhibition) *vs*. standard concentration (between 0.0073 and 60 mg L^{-1}) were obtained. Equations of curves, correlation coefficients, and the linear ranges were presented in Table 2. The AA values of analyzed extracts were reported as equivalents (µg mL^{-1} extract), gallic acid (GAE), caffeic acid (CAE), morin (ME), quercetin (QE), and rutin (RE).

Total phenols content (TPC) determination

TPC of plant extracts was determined according to the spectrophotometric Folin-Ciocalteu method (FCM) (Lungu et al., 2010; Nicola et al., 2020; Popa et al., 2012).

Sample preparations. Extracts were prepared according to the previously working procedure. Prior to analysis, the extracts were appropriately diluted with 96% ethanol. From the 200 mg L^{-1} stock solutions the working solutions were prepared by diluting the stock solutions with the same solvent (96% ethanol), so that the concentrations of the solutions used for drawing the calibration curves were 5 - 150 mg L^{-1}.

Samples were prepared by mixing 125 µL of alcoholic extract/standard with 1250 µL aqueous solution of Folin-Ciocalteu reagent (10% v/v) and 1000 µL sodium carbonate solution (1 mol L^{-1}). Mixtures were allowed to stand at room temperature for 15 min. and absorbance against blank (96% ethanol) was measured in a 1-cm glass test tube. The wavelengths at which the determinations were made are those at which the maximum absorption of the reaction products formed by the Folin-Ciocalteu reagent with the standard antioxidant was recorded. From the recorder variation of the absorbance with the wavelength (200 - 1000 nm), the maximum absorption was found to be 761 for both chlorogenic and caffeic acids. It was worked with 200 mg L^{-1} standard solutions. Calibration curves absorbance *vs*. concentration of standards were drawn. Equations of curves, correlation coefficients, and the linearity domains were presented in Table 2. The results were expressed as equivalents standard (mg L^{-1}).

Total flavonoid content (TFC) determination

The TFC was determined using the aluminum chloride colorimetric method (Lungu et al., 2010, Popa et al., 2012). 250 µL extract (prepared according to the previously working procedure) or standard solution were mixed with 50 µL of 10% w/v aluminum chloride, 50 µL of 1 M potassium acetate and 1.4 mL of distilled water. After the 30 min at room temperature, the absorbance of the reaction mixture was determined at 416 nm, in a 1-cm glass test tube, against 96% ethanol. This value represent the maximum absorption which was found from the variation of the absorbance with the wavelength (200 - 1000 nm), standard concentration = 100 mg L^{-1}. Concentrations between 10 and 160 mg L^{-1} of morin, quercetin and rutin standard solutions (prepared by appropriate dilution of the 200 mg L^{-1} stock solutions with 96% ethanol) were used for calibration curves.

The equation curves, correlation coefficients and linearity domains of obtained curves were presented in Table 2. The TFC was expressed as equivalents standard (mg L^{-1}).

Total anthocyanins (TA) content determination

Vanillin colorimetric assay was used for anthocyanins content determination (Lungu et al., 2010). 250 µL of extract/standard solution were mixed with 625 µL of 1% (w/v) vanillin and 625 µL of 9.0 N HCl in 96% ethanol. After incubation at room temperature for 30 min, absorbance was measured against ethanol 96% in a 1-cm glass test tube at 536 nm (determined by recording the spectrum of the absorbance as a function of wavelength). Cyanin chloride (0.1 mg mL^{-1} in 96% ethanol) was used as standard. Cyanidin concentration of each extract (it is noted c_{sample}) was measured using the relation (2).

$$c_{sample} = (A_{sample} / A_{standard}) \times c_{standard} \quad (2)$$

where $A_{standard}$ = the absorbance of the standard solution;
A_{sample} = the absorbance of the standard solutions/extracts;
$c_{standard}$ = the concentration of standard.

The results are given in mg cyanidin equivalents (CyE) per mL extract.

Statistical analysis

All the experiments were performed in triplicate and the obtained data were expressed as the mean ± standard deviation with the Microsoft Excel Program (Redmond, WA, USA).

3. Results and discussion

To identify *the polyphenols by HPLC-DAD* method, the wavelengths that were followed were: 230 nm, 250 nm, 280 nm, and 300 nm. The chromatographic method allowed identifying the *p*-coumaric acid, caffeic acid, chlorogenic acid, rutin, and epicatechin.

To determine *the antioxidant activity* using spectrophotometric DPPH· method, calibration curves percentage of inhibition (%I) *vs*. concentration of two polyphenolic acids (caffeic and gallic acids) and

three flavonoids (morin, quercetin and rutin, a glycoside of the quercetin) were drawn. As can be seen from Table 1, there is a very good linearity of the presented curves obtained by DPPH· method (r^2 = 0.9939 - 0.9972).

Table 2 shows that the AA values determined for all alcoholic extracts expressed in µg standard equivalents mL^{-1} varies between 1,92 ± 0,17 and 65,8 ± 2,0. *Lonicera* fruit extracts have a higher antioxidant capacity than *Aronia* and *Sambucus* fruit extracts. It is also observed (Table 2) that the extracts are rich in flavone-type polyphenols.

Total phenols content (TPC) was calculated from the equations of the calibration lines, which are presented in Table 1. As can be seen from Figure 1A, TPC values expressed as chlorogenic acid varies from 590.8 ± 62 to 5440 ± 512 mg L^{-1} and between 297.7 ± 29 to 2671 ± 253 mg L^{-1} caffeic acid equivalent. The phenol content expressed in chlorogenic acid (identified by HPLC-DAD as a widespread compound in analyzed extracts) equivalents is much higher than if it was expressed in caffeic acid equivalents for all the extracts analyzed.

The content of flavonoids and anthocyanidins were illustrated in figure 1B and, respectively, 1C. As can be seen in figure 1B, all the studied fruits are rich in flavonoids, but elderberries are distinguished. The flavonoid content varied between 122 ± 11 and 504 ± 19 mg L^{-1} ME, 84.9 ± 7.9 and 351 ± 13 mg L^{-1} QE, 92.3 ± 9.1 and 401 ± 16 mg L^{-1} RE. The highest values were calculated for elderberries extracts obtained by the UAE and the lowest values- for MAE extracts from Honeysuckle berries.

As can be seen in figure 1C, TA varied between 0.653 ± 0.067 and 1.54 ± 0.11mg mL^{-1}, exception of the extract from Elderberries by maceration, with 4.30 ± 0.15 mg cyanidin equivalents (CyE) mL^{-1} extract. The extractions obtained by continuos and ultrasonically assisted methods have the approximately equal values. The elderberries extracts are the richest in polyphenols, of all the analyzed extracts (figure 1 A-C).

The results in this study agreed with literature reports. *Aronia* is a rich source of proanthocyanidins, anthocyanins, flavones, flavonols, polyphenolic acids, especially hydroxycinnamic acids (Denev et al., 2018, Sidor and Gramza-Michałowska, 2019). TPC of some *Aronia* samples was 1022 - 1795 mg $(100g)^{-1}$ FW and total content of proanthocyanidins was in the range 522–1002 mg $(100g)^{-1}$ FW (Denev et al., 2018). The content of hydroxycinnamic acids in the fruits of *Aronia melanocarpa* was almost equal to that of anthocyanins (Denev et al., 2018). For example, chlorogenic acid has reached 187.9 mg $(100\ g)^{-1}$ FW and is in agreement with our study and other studies (Rop et al., 2010).

The total hydroxycinnamic acid determined for *Lonicera* fruits in study (Ochmian et al., 2010) ranged from 22,36 to 30,37 mg $(100\ g)^{-1}$.

In our study, the *Sambucus f*ruits shown an %I between 71.39 ± 5.29 (MAE extract) and 89.38 ± 1.68 for extract obtained by continuous method (data not show). Młynarczyk et al. (2018) report that the antioxidant activity of analyzed elderberry varies from 82.08 to 89.25% of inhibition in relation to the DPPH·. The antioxidant properties of these fruits are primarily attributable to the presence of phenols (Młynarczyk et al., 2018).

The content of biological compounds varies with enviromental factors, harvest date and genotype (Poll and Petersen, 2003, Reyes-Carmona et al., 2005, Hoppula and Karhu, 2006). Research in the field of phenols extracted from plants (Schaffer et al., 2005) shows that phenols depend quantitatively and qualitatively on genetic information (species, variety), environment and geographical conditions. Climate, season, light, temperature, maturation period strongly influence the synthesis of phenols in plants (Aherne and O'Brian, 2002). Studying two species cultivated by *Lonicera*, the authors of the study (Ochmian et al., 2010) showed that early honeysuckle berries have a lower TP content compared to late harvest berries, when there were increases of 65% compared to the initial values. Early fruit of the "Wojtek" variety had a TPC of 174.95 mg $(100\ g)^{-1}$ and of the "Brązowa" variety 144.84 mg $(100\ g)^{-1}$ (Ochmian et al., 2010).

4. Conclusions

The antioxidant activity and phenolic profile of fruits belonging to three cultivar species in Romania were studied**:** *Aronia melanocarpa* 'Melrom' cv., *Lonicera caerulea* var. *kamtschatica*, 'Kami' cv., and *Sambucus nigra* L., 'Elrom' cv. Alcoholic extracts of these berries were obtained by continuous, maceration, ultrasonically assisted extraction and microwaves-assisted extraction. By chromatographic method have been identified in extracts the phenolic acids (*p*-coumaric, caffeic, chlorogenic acids), rutin (a glycosilate flavonoid), and epicatechin. Free DPPH radical scavenging activity of extracts, expressed as equivalents gallic acid, caffeic acid, morin, quercetin, and rutin, points out that the extracts are rich in flavonoids. Total phenols, flavones and anthocyanidins content values confirm this experimental conclusion.

The experimental results have shown that analyzed fruit extracts have high antioxidant activity, due mainly to flavonoids and anthocyanins. In the future we intend to continue these studies and to capitalize

on the very high antioxidant potential of these fruits, introducing them in nutraceuticals with high biological potential (antioxidant, anti-stress, anti-aging activities).

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Tables and Figures

Table 1. Equations, correlation coefficients, and linear range of obtained calibration curves

Analytical method	Standard	Linear equation	r^2/n^a	Linearity domain (mg L^{-1})
DPPH· method	Caffeic acid	$y^b = 5.528x^c + 49.66$	0.9951 / 9	0.030 - 7.50
	Gallic acid	y = 11.14x + 50.03	0.9939 / 8	0.030 – 3.75
	Morin	y = 0.691x + 47.47	0.9972/11	0.015 - 60
	Quercetin	y = 1.18x + 45.60	0.9939 / 7	0.47 - 30
	Rutin	y = 1.44x + 41.48	0.9948 / 7	0.47 - 30
FCM[d]	Chlorogenic acid	$Y^e = 0.0021x^e - 0.0384$	0.9914 / 8	40 - 150
	Caffeic acid	Y = 0.0045x - 0.0483	0.9907/11	10 – 150
Colorimetric, with $AlCl_3$	Morin	Y = 0.0068x - 0.0218	0.9986 / 8	10 – 120
	Quercetin	Y = 0.0097x - 0.0179	0.9960 / 7	20 – 100
	Rutin	Y = 0.0079x - 0.0296	0.9976 / 7	20 – 100

[a]n: number of determinations; [b]y: I (%); [c]x: standard concentration (mg L^{-1}); [d]FCM: Folin-Ciocalteu method; [e]Y:absorbance.

Table 2. Values of DPPH· radical scavenging activity of analyzed fruit extracts

Sample	Extraction method	Free DPPH radical scavenging activity (µg mL^{-1})				
		CAE[e]	GAE[f]	ME[g]	QE[h]	RE[i]
Chokeberries	MAC[a]	4,80 ± 0,23[j]	2,35 ± 0,11	41,5 ± 1,8	25,9 ± 1,1	24,1 ± 0,88
	SOX[b]	4,25 ± 0,13	2,07 ± 0,066	37,2 ± 1,1	23,4 ± 0,63	22,0 ± 0,52
	UAE[c]	5,34 ± 0,56	2,62 ± 0,28	45,9 ± 4,5	28,5 ± 2,6	26,2 ± 2,1
	MAE[d]	5,76 ± 0,10	2,82 ± 0,049	49,2 ± 0,79	30,4 ± 0,46	27,8 ± 0,38
Honeysuckle berries	MAC	7,43 ± 0,089	3,65 ± 0,044	62,6 ± 0,71	38,3 ± 0,42	34,2 ± 0,34
	SOX	7,29 ± 0,37	3,58 ± 0,18	61,5 ± 2,9	35,6 ± 1,7	33,7 ± 1,4
	UAE	7,03 ± 0,18	3,45 ± 0,088	59,4 ± 1,4	36,4 ± 0,83	32,7 ± 0,68
	MAE	7,83 ± 0,25	3,85 ± 0,12	65,8 ± 2,0	40,1 ± 1,2	35,8 ± 0,96
Elderberries	MAC	5,48 ± 0,097	2,68 ± 0,048	47,0 ± 0,77	29,1 ± 0,45	26,7 ± 0,37
	SOX	7,19 ± 0,30	3,53 ± 0,15	60,7 ± 2,4	37,1 ± 1,4	33,3 ± 1,2
	UAE	5,80 ± 0,25	2,84 ± 0,12	49,6 ± 2,0	30,6 ± 1,2	28,0 ± 0,95
	MAE	3,93 ± 0,35	1,92 ± 0,17	34,6 ± 3,1	21,9 ± 2,0	20,8 ± 1,86

[a]MAC: maceration, [b]SOX: continuous (Soxhlet) extraction, [c]UAE: ultrasonically assisted extraction, [d]MAE: microwave assisted extraction; [e]CAE: caffeic acid equivalents; [f]GAE: gallic acid equivalents; [g]ME: morin equivalents; [h]QE: quercetin equivalents; [i]RE: rutin equivalents. [j]Results are the mean of three determinations ± SD (standard deviation).

 DOI 10.33045/fgr.v38.2022.13

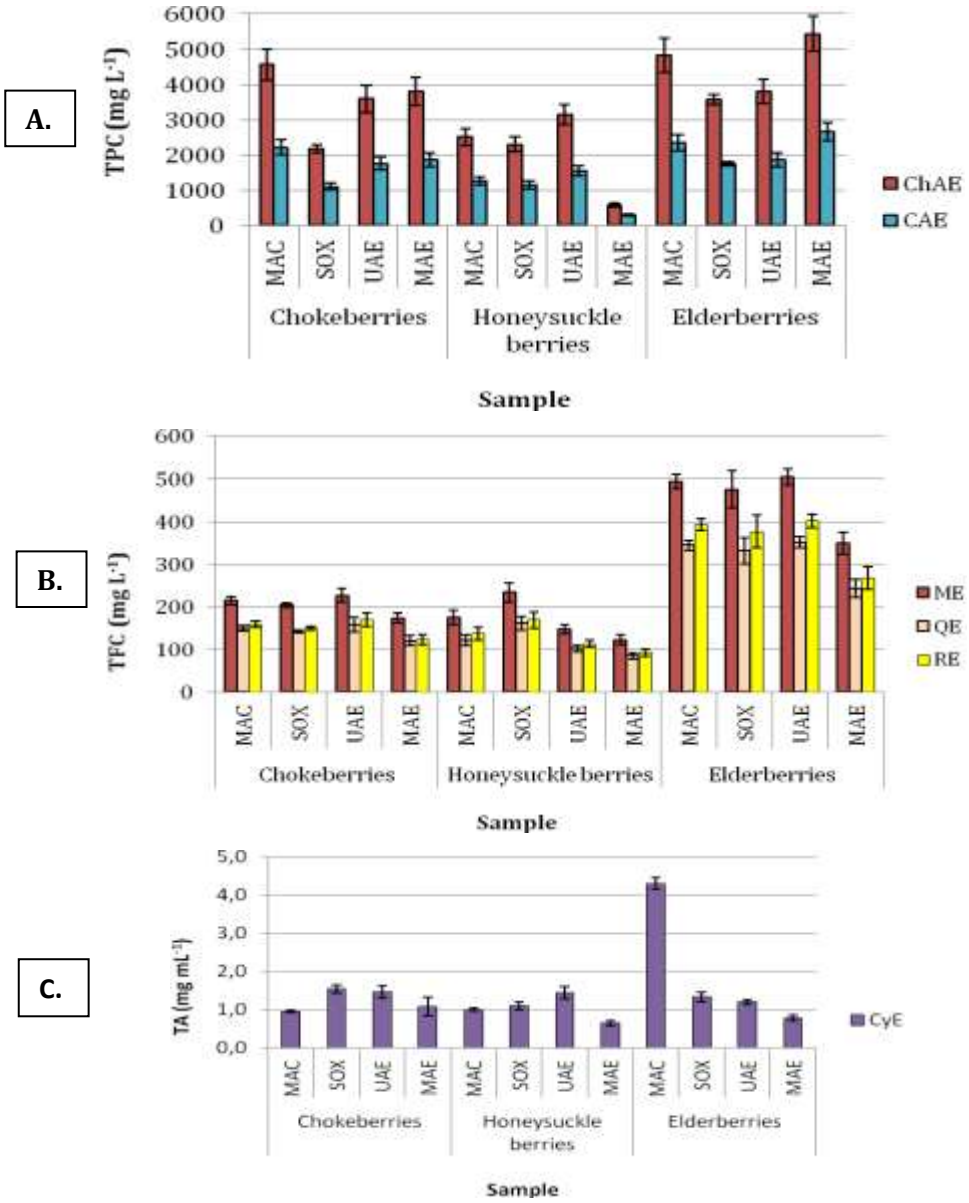

Fig. 1. Content of: A. total phenols; B. flavonoid and C. anthocyanins from the analyzed fruit extracts. MAC: maceration, SOX: continuous (Soxhlet) extraction, UAE: ultrasonically assisted extraction, MAE: microwave assisted extraction; ChAE: chlorogenic acid equivalents; CAE: caffeic acid equivalents; ME: morin equivalents; QE: quercetin equivalents; RE: rutin equivalents; CyE: cyanidin equivalents. Error bars represent ± SD (standard deviation, n = 3).