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COMPARATIVE ANTIOXIDANT ACTIVITY OF SOME *PRUNUS* GENUS FRUITS

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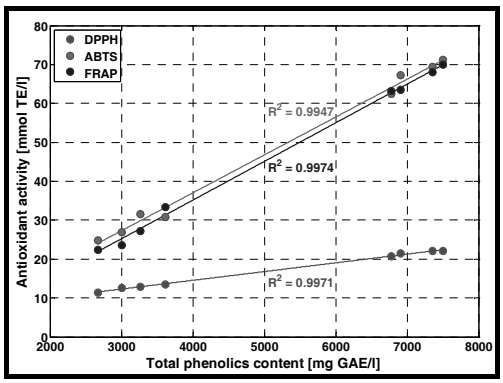
The alcoholic extracts of eight fruits from *Prunus* genus from Banat county of Roumania were analyzed for anthocyanins composition, anthocyanins content, total phenolics, and antioxidant capacity. Extraction of the pigments was carried out with acidified methanol in ultrasonic conditions, 60 minutes at 25°C and 59 kHz. The extracts have been analyzed by high-performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 apparatus equipped with photodiode array detector and a C18 reversed-phase column for anthocyanins separation. The amount of monomeric anthocyanins was determined by using the pH differential method and the total phenolics content was quantified by using the Folin-Ciocalteau method. Evaluation of antioxidant activities of extracts was performed by using FRAP – ferric reducing/antioxidant power assay, ABTS$^{\bullet+}$ – radical-scavenging activity and DPPH – radical-scavenging activity assays. The antioxidant activities of the extracts were correlated with their anthocyanins and total phenolics content. A significant level of anthocyanins (4100-4300 mg/L) and total phenolics (6900-7300 mg GAE/L) contents have been obtained for cherry extracts.



INTRODUCTION

In the last 10 years, many publications have referred to the possible health-promoting effects of anthocyanins and other phenolic compounds presented in fruits and vegetables.[1-3] There are several reports focused on the effect of anthocyanins in cancer chemoprevetion,[4-8] diabet and improvement of vision, [9] cardiovascular diseases,[10] human nutrition,[11-13] biological activity,[14] absoption and metabolism of anthocyanins,[15] and their antioxidant activity.[16]

The current trend of limiting the use of synthetic dyes led to increased interest in natural colorants including anthocyanins. The use of anthocyanins from natural sources as food colorants is widely permitted in Europe, Japan, United States, and many other countries.[3] In the European Union, anthocyanin-derived colorants are recognized as natural colorants under classification E163.[17] Besides their importance as water-soluble natural food colorants, interest for anthocyanins has increased because of their potential health benefits as dietary antioxidants. Identification of anthocyanins is quite complicated due to the large number of anthocyanins found in nature and also due to the lack of standards for many of them. The most efficient method for the qualitative and quantitative analysis of anthocyanins is high performance liquid chromatography (HPLC) coupled with electrospray ionization mass

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spectrometry (MS), especially the tandem mass spectrometry (MS/MS)[18] which can provide mass spectra of molecular ions and fragment ions. Diets rich in fruits and vegetables significantly reduce the incidence and mortality rates of cancer, cardiovascular disorders, and other degenerative diseases caused by oxidative stress.[19, 20]

Oxidation process represents an essential part of our metabolism. Reactive oxygen species - ROS and radicals (O_2^-, HO·, HOO·, lipid radicals L·, LOO·, alkoxyl radicals RO·, ROO·, NO·, NO^+, RS·, protein radicals P·) are continuous produced by the body's normal use of oxygen in respiration and in some cell-mediated immune functions.[21] Higher ROS concentrations than physiological necessary can stimulate free-radical chain reactions and subsequently damage the cellular biomolecules such as proteins, lipids, nucleic acids, polyunsaturated acids and carbohydrates. Anthocyanins and other phenolics have shown to be potent natural antioxidants which can blocked some damages.

Currently, a large variety of analytical methods for assessing antioxidant capacity of foods and biological systems is available. These methods differ in terms of reaction mechanisms, oxidant and target species, reaction conditions, and expression of results. Because of the diversity of testing methods and therefore the difficulty of comparing results between different studies, some authors have suggested the need for standardized testing protocols to measure antioxidant capacity. [22] Taking into account the lack of standardized methods and considering the limitations of various methods[22] it is recommendable to use more than one method for estimating antioxidant capacity of natural antioxidants. This study has focused on comparison of antioxidant activities (AA) of some fruits belonging *Prunus* genus from Banat County, Roumania, correlated with monomeric anthocyanins content – MA and total phenolics content – TP.

RESULTS

Three of the most commonly used methods (DPPH, ABTS, and FRAP) for assessing antioxidant activity of fruits extracts were chosen because a single test does not accurately reflect the antioxidant capacity of a sample. The highest antioxidant activities (AA) were manifested by the sour cherry, bitter cherry and cherry plum and were presented in Table 1, Fig. 1 and Fig. 2.

Anthocyanins HPLC profiles for the most active fruit extracts and a part of MS spectra for identified compounds were presented in Table 2 and Fig. 3.

DISCUSSION

Antioxidant activities assessed by ABTS and FRAP methods showed comparable values, and generally were 2-3 times higher than DPPH values (Table 1, Figs. 1 and 2). The lower values obtained by DPPH method could be a consequence of color interference between the extracts containing anthocyanins and DPPH in the visible region at around 515 nm, and thus the antioxidant activity measured by this assay is underestimated. It can be observed that the correlation of anthocyanins content with antioxidant activities of studied fruits was encouraging for researchers ($R^2 > 0.77$). The best results were obtained when the antioxidant capacity of fruits was related to the total phenolic contents ($R^2 > 0.99$).

Table 1

Monomeric anthocyanins content, total phenolics content and antioxidant activities of the extracts

Fruits	MA [mg/L]	TP [mg/L]	AA[mmol TE/L]			MA/TP
			DPPH	ABTS	FRAP	
Sweet cherry	4371.7 ± 15.8	6905.9 ± 73.0^b	21.5 ± 0.4^b	67.2 ± 2.1	63.6 ± 0.7	0.63
Sour cherry	2702.3 ± 12.6	7499 ± 86.1^a	22.1 ± 0.1^b	71.2 ± 4.7	70.1 ± 0.9	0.36
Bitter cherry	4097.4 ± 3.4	7352 ± 56.8^a	22.1 ± 0.2^b	69 ± 2.5	68 ± 0.6	0.56
Plum	1601.3 ± 11.4	3616.4 ± 32.7	13.5 ± 0.9^a	30.7 ± 0.6	33.4 ± 0.5	0.44
Red cherry plum	233.8 ± 1.2	3266.8 ± 61.2	12.9 ± 0.1^a	31.6 ± 1.8	27.1 ± 0.5	0.07
Cherry plum	2968.8 ± 2.4	6774.9 ± 7.8^b	20.7 ± 1.3^b	62.4 ± 0.7	63.2 ± 0.6	0.44
Peach	1048.5 ± 4.5	3003.4 ± 40.1	12.6 ± 0.6^a	26.9 ± 0.9	23.6 ± 0.3	0.35
Nectarine	1199.1 ± 0.6	2667.5 ± 28	11.3 ± 0.2^a	24.8 ± 0.2	22.3 ± 0.4	0.45

* Data were calculated as mean ± standard deviation (SD). The results were processed by using ANOVA one-way analysis of variance. Differences at $p < 0.05$ were considered statistically significant.

**All data are expressed as means of three replication ± SD. The values followed by the same letter are not significantly different ($p>0.05$)

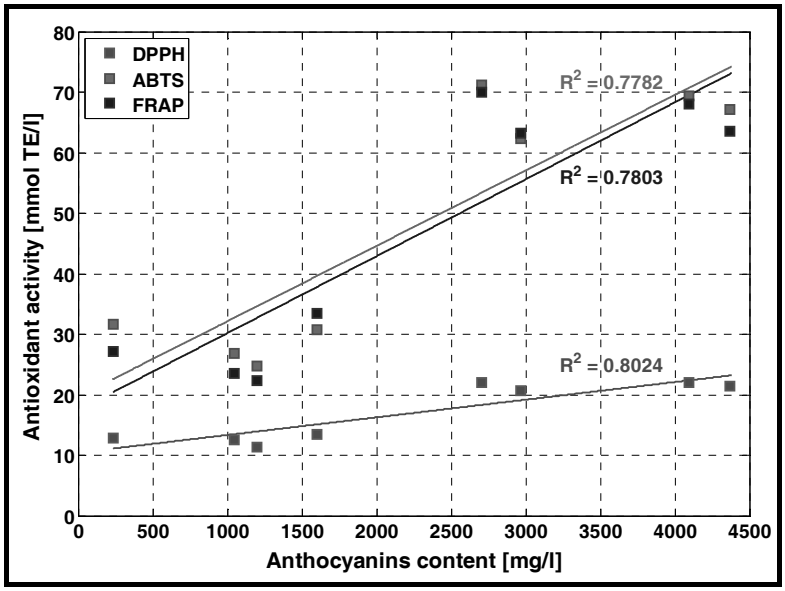

Fig. 1 – Influence of anthocyanins content on antioxidant activity of extracts.

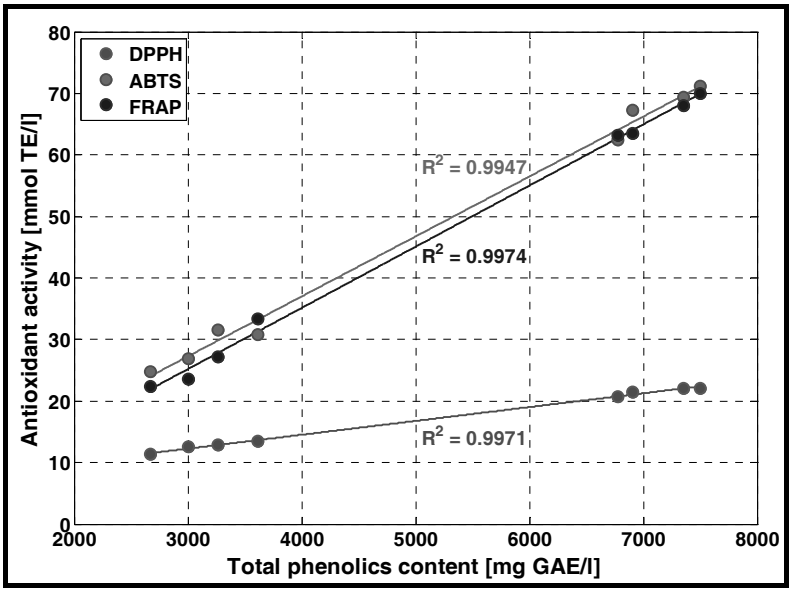

Fig. 2 – Influence of total phenolics content on antioxidant activity of extracts.

Table 2

Mass spectra data of anthocyanins

Peak no.	Anthocyanins	%	Mass	Fragment
	Sour cherry			
1	Cyanidin-3-sophoroside	4.26	611	287
2	Cyanidin-3-glucosylrutinoside	89.43	757	611/287
3	Cyanidin-3-glucoside	0.22	449	287
4	Cyanidin-3-rutinoside	0.35	595	443/287
5	Peonidin-3-rutinoside	5.74	609	463/301
	Bitter cherry			
1	Cyanidin-3-glucoside	0.13	449	287
2	Cyanidin-3-rutinoside	4.10	595	443/287
3	Pelargonidin-3-glucoside	94.78	433	271
4	Pelargonidin-3-rutinoside	0.16	579	433/271
5	Peonidin-3-glucoside	0.70	463	301
6	Peonidin-3-rutinoside	0.12	603	463/301

Table 2 (continued)

Cherry plum				
1	Cyanidin-3-galactoside	0.06	449	287
2	Cyanidin-3-xyloside	25.68	419	287
3	Cyanidin-3-glucoside	12.72	449	287
4	Cyanidin-3-rutinoside	52.95	595	443/287
5	Peonidin-3-glucoside	2.80	463	301
6	Peonidin-3-rutinoside	0.06	603	463/301
7	Pelargonidin-3-glucoside	0.05	433	271
8	*No identified*	0.06	-	-
9	Cyanidin-3-(6”-acetoyl)glucoside	4.55	491	287
10	*No identified*	1.06	-	-

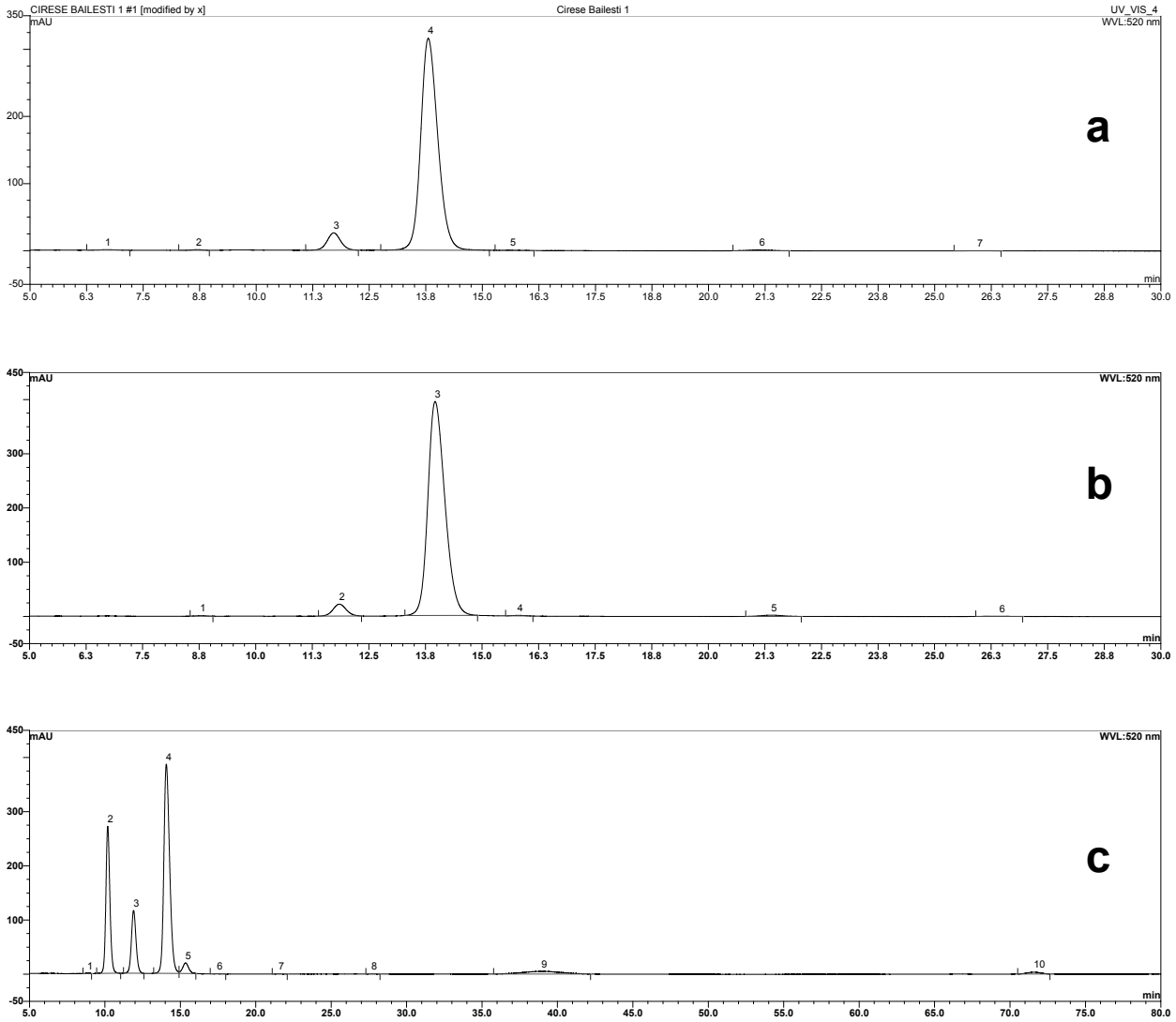

Fig. 3 – HPLC profiles of: (a) Sour cherry; (b) Bitter cherry; (c) Cherry plum.

EXPERIMENTAL

Plant material

Fruits of sweet cherry (*Prunus avium* L.), sour cherry (*Prunus cerasus* L.), bitter cherry (*Prunus emarginata* L.), plum (*Prunus domestica* L.), red and black cherry plum (*Prunus Cerasifera* L.), peach (*Prunus persica* L.) and nectarine (*Prunus persica* L. var. *nucipiersica*) from *Prunus* genus were harvested at the maturity stage during summer 2013 from Banat County, Roumania. After harvesting, the fresh fruits were manually peeled and the skins were cut into small pieces.

Standard and reagents

The anthocyanin standards: cyandin-3-glucoside chloride, cyandin-3-rutinoside chloride, malvidin-3-glucoside chloride, malvidin-3-galactoside chloride, pelargonidin-3-glucoside

chloride, delphinidin chloride, cyanidin chloride and malvidin chloride, Folin-Ciocalteu reagent, 1,1-diphenyl-dipicrylhydrazyl (DPPH), gallic acid (GA), trifluoroacetic acid (TFA), 6-hidroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (Trolox) and methanol (HPLC grade) were purchased from Sigma-Aldrich, Germany. Iron (III) chloride, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchase from Sigma, Germany. Hydrochloric acid was purchased from Merck, Germany. Ultrapure water for lab analyses was obtained by an EASYpure® RoDi Barnstead system (USA).

Extraction and samples preparation

Extractions of anthocyanins were made as followed: 10 g skins of each fruit were mixed with 0.1% HCl in methanol (solid to solvent ratio 1:4 (w/v) and sonicated at constant frequency 59 kHz, 60 min., at room temperature. The extracts were concentrated under vacuum at 35-40°C in a rotary evaporator (Laborota 4000 Efficient, Heidolph, Germany) until complete solvent evaporation. All extracts (brought up to a volume of 10 ml with methanol) were vortexed and centrifuged (20 min., 2000 *x g*) using a Labnet C1301 centrifuge (Labnet International, Korea).

Determination of anthocyanins content

The monomeric anthocyanin content was quantified by the pH differential method.[23] The absorbances at $\lambda_{vis\text{-}max}$ and 700 nm were read using a Jasco V 530 UV-Vis spectrophotometer (Abble &Jasco, Germany). The monomeric anthocyanins concentration was expressed as cyaniding-3-glucoside equivalents in mg of anthocyanins/L of fruit extract. All experiments were realized in triplicate.

Determination of total phenolics content

The content of total phenolics was determined according to the Folin-Ciocalteu method,[24] using gallic acid as standard. The calibration curve of gallic acid was obtained using 10 standard solutions in the range 50-550 mg/L; total phenolics content was expressed as mg GAE/L. All experiments were realized in triplicate.

In vitro evaluation of antioxidant properties

DPPH radical-scavenging activity

The free radical scavenging activity of the extracts was performed by using DPPH,[25] with some modifications. Each sample has been diluted 1:20 v/v with methanol, then 100µL was added to 2.9 mL of a solution ~$9 \cdot 10^{-5}$ mol/L DPPH in methanol. The inhibition of DPPH was followed by monitoring the decrease of absorbance at 515 nm during 2 hours. The inhibition of DPPH was expressed using Trolox as antioxidant reference compound ($I\ [\%] = (A_0 - A_{t=2h}) / A_0 \cdot 100$). All experiments were realized in triplicate.

$ABTS^{\cdot+}$ radical-scavenging activity

The ABTS (7 mmol/L in 20 mmol/L sodium acetate buffer, pH=4.5) reacts with potassium persulfate, 2.45 mmol/L in same solution.[26] The dark blue-green stable radical solution resulted is incubated 16-18 h, at room temperature, in the dark. The reaction between mixture of 100µL sample and 2.9 mL ABTS reactive, was followed at 734 during 2 hours, against dd water (the antioxidant reference compound was Trolox). All experiments were realized in triplicate.

FRAP-Ferric reducing/antioxidant power assay

The fresh FRAP solution was prepared by mixing 300 mmol/L acetate buffer pH=3.6 (3.1 g $CH_3COONa \cdot 3H_2O$ and 16 ml acetic acid) with 10 mmol/L TPTZ in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \cdot 6H_2O$ in dd water in vol. 10:1:1 ratio.[27] The resulting solution was diluted with 2 volumes of dd water and was incubated at 37°C for 30 min. 2.9 mL of working FRAP solution were mixed with 100 µL of extract, was kept in dark for 2 hours, at room temperature. The absorbance of the samples and a blank was measured at 593 nm against dd water using a Jasco V 530 UV-Vis spectrophotometer (the antioxidant reference was Trolox). All experiments were realized in triplicate.

HPLC analysis of anthocyanins

A Dionex Ultimate 3000 apparatus (Dionex Corporation, USA) equipped with a LPG 3400A quaternary pump, PDA 3000 photodiode array detector, and thermostated column compartment TCC-3000 was used.[35] C-18 Acclaim® 120 Silica-Based reversed-phase column (5µ, 4.6 x 150 mm, Dionex Corporation) and a mobile phase: aqueous solution of 30% methanol containing 0.5% TFA (solvent A) and methanol (solvent B), with constant flow rate of 1.0 mL/min were chosen for anthocyanin separations. The process was governed by software Chromeleon 6.8.

MS analyses

A high capacity ion trap instrument HCT MS Bruker Daltonics (Bremen, Germany), coupled with the NanoMate robot (Advion Biosciences, Norfolk, UK) for automatic infusion of samples by chip-electrospray was used. The work conditions were: positive ion mode, 50-2800 *m/z* range, scan speed of 8000 *m/z* per second, nitrogen as nebulizer gas (50 psi), helium as collision gas and the ESI initiation was 1.4 kV on the conductive pipette tip of the NanoMate and (-50 V) on the HCT counter-electrode. We used softwares Esquire Control 6.1.512 for HCT MS instrument and ChipSoft 7.1.1 for NanoMate robot. [28]

CONCLUSIONS

This work wants to encourage the consumption of fresh *Prunus* genus fruits for beneficial effects on preventing some diseases. Thus, the current study highlights the difference in total phenolic and anthocyanin contents, and antioxidants capacity found in different *Prunus* genus fruits grown in Banat County, Roumania. The sweet, sour and bitter cherries and cherry plums showed high contents of anthocyanins and phenolics and higher antioxidant activities, respectively. Total phenolics content of the extracts are highly correlated with their antioxidant activity assessed by the three methods ($R^2 > 0.99$). In studied series the antioxidant capacity decreases in order: sour cherry > bitter cherry > sweet cherry > cherry plum > plum > red cherry plum > peach > nectarine.

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