Studies on the Pholyphenolics Compounds Extraction from Vaccinium Fruits

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Bilberries are known for their bioactive properties such as antioxidant activity. They contain a wide range of flavonoids and phenolic acids that show antioxidant activity. In the present work, the total phenolic and flavonoid contents, correlated with antioxidant activity of Vaccinium extracts were determined. The content of total phenolics in the extracts was determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents (GAE). The content of total flavonoids was measured also spectrophotometrically by using the aluminum chloride colorimetric assay. Effects of ethanol and methanol concentrations of the solutions on the contents of flavonoids and phenolic in extracts were investigated.

Keywords: total phenolics, total flavonoids, antioxidant activity, DPPH

Over the past years, researchers and food manufacturers have become increasingly interested in polyphenols. The main reason for this interest is the recognition of the antioxidant properties of polyphenols, their great abundance in our diet, and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular diseases [1].

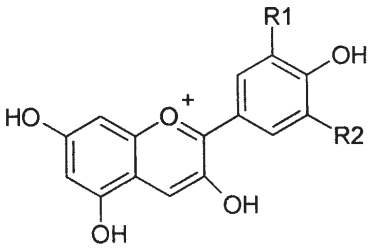
Vegetable tissues contain lots of substances having phenolic functional groups that belong to different classes of organic compounds. It may be difficult to classify them, because most of the natural compounds contain in the same time more types of functional groups. The classification of the phenolic compounds that is based on the carbon skeleton structure was proposed by Harborne in 1964 and it is used until now, with some changes [2].

Fruits, legumes, cereals have been extensively explored because these products are “basic foods” in the human diet. Active antioxidant components in these sources are “secondary metabolits”, polyphenolic compounds as: phenolic acids, flavonoids, anthocyanidins, tocols, carotenoids, ascorbic acid etc., compounds with antioxidant activity [3]. For this reason, in the latest years the interest of changing the synthetic antioxidants with natural ones from vegetable sources has increased.

An antioxidant may be defined as “any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly, delays or inhibits oxidation of that substrate”. The main characteristic of an antioxidant is its ability to trap free radicals [4].

Many type of fruits, like bilberry, blackberry, gooseberry, from berries fruits class, have a high content of flavonoids and polyphenols, compounds with recognized antioxidant activity. This property is due to the hydroxy groups, which can neutralize the oxygen radicals (responsible for initiating and propagating the oxidation reactions) resulting more stable compounds [5]. Main flavonoid subgroup in berries and fruits are anthocyanins. Lowbush and highbush blueberries have an unusual content of significant amounts in the five aglycones: delphinidin, cyanidin, petunidin, peonidin and malvidin [6].

Anthocyanins, are pigments which belong to the secondary metabolite group of flavonoids, and are often responsible for the orange, red and blue colors in fruits,



Antocyanidin	R1	R2
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Fig. 1. Structural formulas of common anthocyanins

vegetables, flowers and other storage tissues in plants. Thus, they have become important as food additives. Bilberry fruits (Vaccinium myrtillus and Vaccinium vitis-idaea) are used as natural colorants in some sweet food products and it is expected that their extracts show some antioxidant activity, because of their composition [7].

The antioxidant activity of vegetable extracts depends on a proper solvent selection for extraction and isolation of all the active principles. The most common solvents used for the extraction of polyphenolics from plant material are methanol, ethanol, acetone, ethyl acetate, and their aqueous solvents [8].

Searching for an efficient method for extracting phenols from Vaccinium species fruits have been of great interest, due to the health promoting and disease preventing effects of polyphenols. In this study, extractions were performed by using aqueous ethanol and methanol. The effects of single factors such as the concentration of ethanol (methanol) on the efficiency were studied.

The goal of this study was to investigate the effect of extracting with different concentration solvents, on total phenolics, total flavonoids and antioxidant activity of Vaccinium extracts. Antioxidant activity of Vaccinium extracts was compared with that of standard solution of anthocyanins and butylated hydroxytoluene (BHT).

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Materials and methods

Vaccinium myrtillus fruits (bilberry dried fruits) and *Vaccinium vitis-idaea* juice fruits (mountain cranberry fruits); solution of standards polyphenols (gallic acid, catechin, anthocyanins (Delphinidin, Cyanidin, Malvidin, Peonidin, 10^{-4} g/L)), were purchased from Sigma-Aldrich (Germany), methanol p.a., Merck, 96% ethanol was obtained from Chimopar, Bucharest, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), Merck, Folin Ciocalteu phenol reagent for determining total phenolics extracted was purchased from Merck.

Extraction by ethanol/water solution and methanol/water solution at room temperature:

Vaccinium myrtillus dried fruits (2 g) and *Vaccinium vitis-idaea* juice (4 g) were extracted with 1:10 (w/v) aqueous ethanol (ethanol concentration of 20, 30, 50, 70, 80, and 94% by volume) respectively aqueous methanol (methanol concentration of 20, 30, 50, 70, 80 and 96% by volume) in a shaker at 400 rpm at room temperature for one hour. The mixture was centrifuged at 2000 rpm for 6 min and the supernatant was collected, filtered and stored in a freezer at 5^0C for analysis next day. Because polyphenols are extremely sensitive to light, all procedures were conducted under dim light [9, 10].

Determination of total phenolics by Folin Ciocalteu colorimetry

Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin's reagent. Folin-Ciocalteu (FC) colorimetry is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides [8].

In this study, total phenolic compounds were determined with Folin-Ciocalteu reagent using gallic acid as standard. To 0.5 mL of ethanolic (methanolic) solution of extracts, 7 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (Merck) were added and mixed well. After 3 min, 2 mL of 20% sodium carbonate was added and mixed well again. The absorbance of the resultant solution was read at 720 nm, after maintaining 1 h in a water bath at 25^0C. The concentration of total phenolic compounds was calculated with a linear regression equation obtained from gallic acid standard graph and the results were expressed as milligrams of gallic acid equivalents (mg GAE) in to 100 g dried fruits (for *Vaccinium myrtillus* fruits) and respectively in to 1L of juice (for *Vaccinium vitis-idaea* juice) [11].

Total flavonoid assay

Total flavonoid content was measured by the aluminum chloride colorimetric assay. An aliquot (1 mL) of extract was added to 10 mL volumetric flask containing 4 mL of distilled water. To the flask 0.3 mL 5% $NaNO_2$ was added. After 5 min, 0.3 mL 10% $AlCl_3$ was added. After 6 min, 2 mL 1M NaOH was added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against blank reagent at 510 nm. Total flavonoid content of fruits and vegetables was expressed as mg catechin equivalents (mg CE)/100 g vegetable mass, using a calibration curve [12, 13].

DPPH free radical-scavenging assay [14,15]

The antioxidant activity of Vaccinium extracts was measured by the spectrophotometric method (λ=517 nm), which uses 1,1 diphenyl-2-picrylhydrazyl (DPPH) free radicals. 0.25 mL of the sample was added to 4 mL of DPPH (10^{-4} g/L) solution. When DPPH reacts with an antioxidant compound donating hydrogen, DPPH is reduced, resulting in a decrease in absorbance at 517 nm. The absorbance was recorded at 5 min up to 60 min intervals using a UV-VIS spectrophotometer. The antioxidant activity commensurate with DPPH percentage was calculated using the next formula:

$$\%\ Antioxidant\ activity = \frac{A_{(t=o)} - A_{(t=60)}}{A_{(t=o)}} \cdot 100$$

where:

$A_{(t=o)}$ = sample absorbance at t=0 min.

$A_{(t=60)}$ = sample absorbance at t=60 min.

Table 1

CONTENT OF TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN ETHANOLIC EXTRACTS FROM *VACCINIUM MYRTILLUS* FRUITS

No.	Solvent used for extraction	Total phenolics (mg GAE/100 g dried fruits)	Total flavonoids (mg CE/100 g dried fruits)	Flavonoids/ Phenolics	Antioxidant activity, %
1.	20% EtOH	258.2	160	0.62	87.26
2.	30% EtOH	354.8	282.1	0.79	87.67
3.	50% EtOH	410.6	359.8	0.87	88.15
4.	70% EtOH	416.8	396.3	0.95	89.32
5.	80% EtOH	716	511.1	0.71	89.73
6.	94% EtOH	683.6	501.8	0.73	88.54

Table 2

CONTENT OF TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN METHANOLIC EXTRACTS FROM *VACCINIUM MYRTILLUS* FRUITS

No.	Solvent used for extraction	Total phenolics (mg GAE/100 g dried fruits)	Total flavonoids (mg CE/100 g dried fruits)	Flavonoids/ Phenolics	Antioxidant activity, %
1	20% MeOH	434.8	292.4	0.67	87.94
2	30% MeOH	535.8	350	0.65	88.21
3	50% MeOH	575.6	409.9	0.71	86.61
4	70% MeOH	670.6	484.9	0.72	86.22
5	80% MeOH	711	533.6	0.75	87.21
6	96% MeOH	714	549.6	0.77	87.37

Statistic analysis methods

Some researchers found a strong correlation among antioxidant capacity, total phenolics and total flavonoids [16].

The results from this study were statistically analized, and the basic statistical measures (R,R^2) were calculated using a computer program-Statistica [17].

Results and discussion

The results for total phenolics, total flavonoids content and the total flavonoids/phenolics ratio in Vaccinium myrtillus fruit extracts are presented in table 1 and 2.

The highest total phenolic content was found in 80% EtOH extract followed by 96% MeOH extract. Comparatively total phenolic content was found in 80% EtOH extract and 80% MeOH extract.

The substantial difference in total phenolic content of 70% EtOH extract and 70% MeOH extract should be emphasized (416.8 mg GAE/100 g dried fruits vs. 670.6 mg GAE/100 g dried fruits) at approximately close total flavonoids values (396.3 mg CE/100 g vs. 484.9 mg CE/100 g dried fruits).

The discussion of this difference would have a higher degree of uncertainty, due to the small number of tests. The best solvent used for phenolic compounds extraction from *Vaccinium myrtillus* dried fruits was proved to be methanol but in food industry the ethanolic extract is more convenient. From this study we observed that the ethanolic extracts have a better antioxidant activity (average >88%).

Table 3 and 4 present the analytical data for total phenolic and total flavonoid content of the ethanolic and methanolic extracts from *Vaccinium vitis-idae*a juice.

In the group of methanolic extracts from *Vaccinium vitis-idaea* juice the flavonoids/total phenolics ratio are approximately equal. In the case of ethanolic extracts, the greater content of total phenolics and total flavonoids was found in 70% EtOH extract, while in the case of methanolic extracts, the 80% methanol were proved to be the best solvent for the extraction of phenolics compounds. The results for 80% MeOH and 96% MeOH are quite similar. Although great quantities of total phenolics were registered in the case of extracts that used methanol, our data suggest richer presence of phenolics in the ethanolic extract at 30-94% concentration of EtOH (>1000 mg GAE/L juice).

In this study we analyzed the antioxidant activity of all studied extracts which was compared to the antioxidant activity of standard anthocyanin solution (10^{-4} g/L) and with antioxidant activity of one synthetic antioxidant (BHT). The different antioxidant activities of the analyzed extracts can be described to their total phenolic and flavonoids concentration.

DPPH radical-scavenging capacity (antioxidant activity) of standard solution and BHT are presented in table 5.

Comparing the values of antioxidant activity (~88% for ethanolic extracts and ~ 87% for methanolic extracts) we can see that the presence of more phenolics (flavonoids, anthocyanins) in the same time in vegetable extracts increases the antioxidant activity compared with that of standard solution.

The effect of the ethanol and methanol concentration showed the increase in flavonoids content. The contents of flavonoids from Vaccinium vitis-idae juice methanolic extract increased slowly when the concentration of MeOH was greater than 70%.

No.	Solvent used for extraction	Total phenolics (mg GAE/100 g dried fruits)	Total flavonoids (mg CE/100 g dried fruits)	Flavonoids/ Phenolics	Antioxidant activity, %
1.	20% EtOH	929.65	252.96	0.27	83.31
2.	30% EtOH	1126.34	320.60	0.28	88.55
3.	50% EtOH	1084.63	329.76	0.30	89.08
4.	70% EtOH	1248.07	411.48	0.33	87.86
5.	80% EtOH	1155.60	375.77	0.32	90.99
6.	94% EtOH	1094.70	359.97	0.33	89.30

Table 3
CONTENT OF TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN ETHANOLIC EXTRACTS FROM *VACCINIUM VITIS-IDAEA* JUICE

No.	Solvent used for extraction	Total phenolics (mg GAE/100 g dried fruits)	Total flavonoids (mg CE/100 g dried fruits)	Flavonoids/ Phenolics	Antioxidant activity, %
1	20% MeOH	857.99	274.82	0.32	87.94
2	30% MeOH	964.44	302.17	0.31	88.21
3	50% MeOH	982.07	321.29	0.32	86.61
4	70% MeOH	1113.93	357.12	0.32	86.22
5	80% MeOH	1153.99	361.58	0.31	88.21
6	96% MeOH	1151.70	369.36	0.32	87.37

Table 4
CONTENT OF TOTAL PHENOLICS AND TOTAL FLAVONOIDS IN METHANOLIC EXTRACTS FROM *VACCINIUM VITIS-IDAEA* JUICE

Standard solution	Peonidin	Malvidin	Delphinidin	Cyanidin	BHT
Antioxidant activity, %	12.32	26.82	78.79	73.27	88.29

Table 5
ANTIOXIDANT ACTIVITY OF STANDARD SOLUTION AND BHT

Statistic analysis

There is a strong correlation between total phenolics and total flavonoids contents and between total polyphenols and concentration of methanol solution used for extraction in Vaccinium extracts (table 6).

The values of the correlation coefficient are acceptable, indicating a suitable correlation by linear regressions, which demonstrate the polyphenolic compounds contribution as active principle at antioxidant activity of studied extracts.

On the basis of the simple regression testing, there was a statistically significant relationship between the total phenolics – total flavonoids and between the total phenolics – methanol concentration in methanolic extracts (R>0.9 compared with 0.8 in ethanolic extracts).

Table 6

CORRELATION BETWEEN TOTAL PHENOLICS AND TOTAL FLAVONOIDS CONTENTS AND BETWEEN TOTAL POLYPHENOLS AND CONCENTRATION OF EXTRACTION SOLUTION

	R – square	P
*Vaccinium myrtillus ethanolic extract**		
Total phenolics – total flavonoids	0.8883	0.0049
Total phenolics – ethanol concentration	0.8166	0.0135
*Vaccinium myrtillus – methanolic extract***		
Total phenolics – total flavonoids	0.9819	0.0001
Total phenolics – methanol concentration	0.9349	0.0016
*Vaccinium vitis-idaea – ethanolic extract**		
Total phenolics – total flavonoids	0.8897	0.0047
Total phenolics – ethanol concentration	(error 0.088)	—
*Vaccinium vitis-idaea – methanolic extract***		
Total phenolics – total flavonoids	0.9805	0.0001
Total phenolics – methanol concentration	0.9201	0.0025

R=correlation coefficient, P=confidence limits, *=extracts with 20,30,50,70,80,94% ethanol
**=extracts with 20,30,50,70,80,96% methanol

Conclusions

This study showed important data regarding the antioxidant activity, total phenolic and total flavonoid content of Vaccinium fruits.

In this study the yield of phenolics and flavonoids extracted was influenced by the concentration of alcohol in water.

To obtain the maximum yield of extraction, 80% aqueous ethanol and 96% methanol is recommended for the extraction of phenolic compounds from Vaccinium myrtillus dried fruits and 70% aqueous ethanol and 80% aqueous methanol from Vaccinium vitis-idaea juice.

Antioxidant capacity is an important fruit quality parameter. The presented data for total phenolic and total flavonoid content are a basis for assessment of the preventive role of this fruits against free radicals effect.

Future plans include chromatographic separation of the antioxidant constituents (anthocyanidin as delphinidin, cyanidin, malvidin) in the studied extracts and their uses as protectors in some oxidizable substrates.

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