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DETERMINING SECONDARY METABOLITES AND THEIR ANTI-OXIDANT ACTIVITY IN FRUITS

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Abstract: The influence of extraction techniques with different solvents on the contents of total phenols, flavonoids and antioxidant activity of extracts of grape, sour cherry and black currant is analyzed in the study. Antioxidant activity of the extracts on stable 1,1-diphenyl-2-picryl hidrazyl (DPPH) radical was determined by spectrophotometry. The total phenolic content was determined by using the Folin-Ciocalteu assay while total flavonoid content was measured spectrophotometrically with the application of $AlCl_3$. The changes in the value of these parameters after deep-freezing of these fruits were also observed.

A high content of total phenols and flavonoids was detected in extracts. Acetone proved to be the best agent for extraction of grape and sour cherry while the highest content of total phenols with black currant was obtained by methanol. The content of total phenols in fruits in frozen state increased while the content of total flavonoids and of antioxidant activity decreased. The obtained results show that the extracts of grape, cherry and black currant can be used as natural antioxidants.

Keywords: extraction, phenols, flavonoids, antioxidant activity.

1. INTRODUCTION

Phenolic compounds represent a widely distributed group of secondary plant metabolites that can be of a very simple structure, such as phenolic acids, or of a very complex structure, i.e. semi-condensed substances such as proanthocyanidins. A common characteristic of phenolic substances is that they contain an aromatic ring with one or more hydroxyl groups. Antioxidant activity of phenols is considered to be primarily a result of their ability to be the donors of hydrogen atoms and as such to remove free radicals while forming less reactive phenoxyl radicals:

$$Ph\text{-}OH + ROO\cdot \rightarrow Ph\text{-}O\cdot + ROOH$$

$$Ar\text{-}OH + \cdot OH \rightarrow H_2O + ArO\cdot$$

The increased stability of phenoxyl radicals is a result of delocalization of electrons and existence of a number of resonant forms [1].

Flavonoids are the most common group of phenolic compounds in plants with 15 carbon atoms in the main C6-C3-C6 structure, of which one belongs to benzopyrene ring, while the remaining six carbon atoms make up a benzene ring, connected with the benzopyrene ring on position two [2,3]. Free radicals are atoms, ions and molecules that have one or more unpaired electrons in their structure. Unpaired electrons are a cause of their high and non-selective activity and instability. Free radicals belong to the most reactive chemical types and due to their high chemical reactivity easily react with other molecules, whereby unpaired electrons form chemical bonds, the energy is released and the system passes to a lower energy condition.

According to the manner of their effect on human organism, they are classified into preventive antioxidants, „scavenger" antioxidants and „reparative" antioxidants [4]. Preventive antioxidants prevent formation of free radicals, while „scavenger" antioxidants have an ability of „catching" the free radicals, and the „reparative" antioxidants either renew or remove the damaged vital biomolecules arising under conditions of oxidative stress. According to the place of formation, antioxidants important for human organism are divided into: endogenous and exogenous. Endogenous antioxidants are those that are formed in human organism, while exogenous oxidants are taken through food or medicaments. One of the most important groups of natural antioxidants includes phenolic compounds the activity of which depends on structural characteristics. Phenolic compounds neutralize free radicals in cells through different mechanisms, they prevent

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oxidative damages of DNA and spreading of tumors [5]

Flavonoid quercetin inhibits cytochrome P450 enzymes, that are proved to encourage bioactivation of carcinogens. Phenolic compounds as antioxidants may be efficient inhibitors of LDL (low-density lipoproteins) oxidation; the current researches show that the intake of food rich in flavonoids causes a decrease of cardiovascular diseases [6,7,8]. All this data shows that the increased consummation of fruits and vegetables that show antioxidant properties may improve the quality of life.

2. EXPERIMENTAL PART

The fruits of black grapes of species *Mirisavka* and the black currant picked during the period of full vegetative maturity in the area of Banja Luka and Laktasi Municipalities were used as a material for detecting secondary metabolites, total phenols and flavonoids. One part of the material was chemically analyzed immediately after picking, while the other part was deep-frozen at −20 ^{0}C and treated after six months in the same way as the fresh prepared samples.

2.1. Reagents and chemicals

Folin-Ciocalteu reagent (Sigma Chemical Company, St. Louis, SAD), gallic acid, catechin, 1,1-dyphenil-2picryhydrazyl (DPPH) and aluminum chloride (Sigma Chemical Company, St. Louis, SAD). All other chemicals are of analytic purity degree (p. a.).

Extraction of phenolic compounds was done by three solvents: 2% HCl solvent in methanol, acetone and ethyl acetate. 1 g of sample was weighed with $\pm$ 0,1 g accuracy in Erlenmeyer flask with rimmed neck with volume of 100 mL, then 40 ml of solvent was added and manually homogenized. It was placed in a water bath at 60 ºC with reflux, for 60 minutes. After completed extraction, the content was filtered through the Buchner funnel in volumetric flask with 50 ml of volume in which solvent was added used for extraction up to the label. The obtained extracts were used to determine total phenols, flavonoids and antioxidant capacity. Two parallel measurings were done on the same sample. Extracts were stored at a dark place at the temperature of 4 ºC until analysis.

Total phenols were determined by Folin-Ciocalteu method [9] which is spectrophotometric and based on phenol group oxidation by adding Folin-Ciocalteu reagents with forming of a coloured product. Phenol groups oxidize up to quinone by adding the mixture of molybdophosphate and wolfram phosphate anions that are reduced and give blue coloring. Non-reduced Folin-Ciocalteu reagent is of yellow colour while the reduced one has stable blue colour. Dying intensity is measured by determining absorbance at 765 nm. The results are expressed in gallic acid equivalents. Based on measured absorbances, the mass concentration (μg/mL) of polyphenol compounds are determined from the calibration curve of standard gallic acid solution, by using the equation of a straight line $A = 0{,}0041 \cdot c + 0{,}0204$ $R^2 = 0{,}998$, and then the contents of polyphenol compounds in initial sample is expressed as gallic acid equivalent.

Flavonoid concentration was determined by using $AlCl_3$ as reagent by spectrophotometric method. One of the characteristics of flavonoids is the one of building appropriate metallic complexes with metals [10]. Complex with Al^{3+} is particularly important. Absorbance was measured at $\lambda = 510$ nm, with regards to deionized water as blank. Based on measured absorbances, the mass concentration (μg/mL) of flavonoid is determined from the calibration curve of standard solution of catechin, by using the equation of a straight line $A = 0{,}0243 \cdot c + 0{,}009$, and then the content of total flavonoid in the initial sample is expressed as catechin equivalent.

Anti-oxidation capacity of tested samples was determined by measuring their capacity to neutralize DPPH$^{\bullet}$ radicals (2,2-diphenyl-1-picrylhydrazyl) which, due to unpaired electron, exhibit strong absorption in the visible part of spectrum [11]. By pairing an electron pair of stable DPPH$^{\bullet}$ radical in the presence of electron donor (antioxidant that catches free radicals), violet colour changes to yellow. The decolorization that occurred is in stoichiometric relation with the number of paired electrons [12]. Absorbances of resulting solutions are determined by spectrophotometry at 515 nm after 30 minutes of staying at room temperature.

Radical Scavenging Capacity-RSC is calculated on the basis of the following equation:

$$\%RSC = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100$$

Based on measured differences of absorbances $\Delta A = A_{blank} - A_{sample}$, from calibrated curve of standard Trolox solution, the total antioxidant activity in μmol/mL was determined by using the equation of a straight line

TAA= 0,1684· ΔA – 0,0048), and then the total antioxidant activity in the initial sample was expressed as trolox equivalent. Trolox is water-soluble derivative of vitamin E. The anti-oxidant potential (AP) was calculated from the relation:

$$AP) = \frac{TAA(mmoli\ trolox)}{TP(mg\ galic\ acid)}$$

TAA – total antioxidant activity
TP – total phenols

3. RESULTS AND DISCUSSION

Chemical composition of fruits is very complex and depends on the species, ecological conditions, maturity degree, applied measurement technique, as well as on many other factors. There are even differences in the chemical composition of the berries of the same type.

Spectrophotometric methods were applied to analyze extracts of the fruit berries for composition and content of polyphenol compounds as well as for their antioxidant activity. Folin-Ciocalteu method was used to determine the contents of total phenols. A disadvantage of the method is in its insufficient specificity, because phenols are detected with different sensitivity. Despite that, this method was used in this study, given the fact that it is a standard method for determining the content of total phenol in tested fruit extracts. The results were expressed as gallic acid equivalents (GAE). The method on DPPH• radicals is widely used to determine „scavenger“ capacity. Antioxidants react with stable DPPH• radicals and transform them. The level of decolorization of solution of DPPH• radicals shows the „scavenger“ potential of antioxidant compound. It was exactly this method that was applied to determine antioxidant activity of extracts of tested fruits. Methanol solution of trolox was used in this test as reference antioxidant. The results obtained from determining total phenols, flavonoids and antioxidant activity of both fresh fruit and after freezing at −20 ºC during six months are presented in Table 1.

Table 1. Content of total phenols (TP), flavonoids (TF) and antioxidant activity (TAA) of extracts of fresh and frozen grapes in different solvents

Solvent	Fresh fruits after picking			Fruits frozen at -20^{0}C for six months		
	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g
methanol	68,4	30,6	0,653	75,4	26,4	0,55
acetone	70,6	33,7	0,670	82,6	30,7	0,65
ethylacetate	65,7	28,3	0,580	70,4	20,8	0,48

TP total phenols expressed as mg equivalents of gallic acid in 100g of fresh or defrosted fruits; TF total flavonoids expressed as mg of catechin equivalents in 100g of fresh or defrosted fruit; TAA antioxidant activity expressed as trolox equivalents.

Based on obtained values of total phenols of grapes we can conclude that acetone proved as the best solvent, as its extraction power was by 7% higher compared to ethylacetate. Acetone extract again had the highest content of total flavonoids and it also showed the highest antioxidant activity. In all extracts of grapes that were previously frozen at −20 ºC the amount of total phenols increased, but this phenol increase was not accompanied by an increase in total flavonoids and antioxidant activity, suggesting that there was a decrease of some substances too (which are most probably thermo-instable), and that have an influence on antioxidant activity of grapes. As with the fresh grape, with previously frozen grape too, acetone had the highest and ethylacetate the lowest extraction power. Lower antioxidant activity of frozen fruits point out at a recommendation for using the fresh fruits.

The content of total phenols, flavonoids and of antioxidant activity with sour cherry in fresh and frozen condition are presented in Table 2.

All extracts of fresh and subsequently frozen sour cherry fruits had higher values of total phenol, flavonoid and antioxidant activity compared to the extracts of grape. This indicates higher antioxidant potential of sour cherry. Like with grapes, acetone proved to be the best extraction solvent, and ethylacetate was the worst in that respect as it contained 13.6% less of total phenols and 14.3% less of total flavonoids. By determining secondary metabolites of defrosted sour cherry fruits an increase in total phenols of 3 to10% was observed again with

defrosted sour cherry fruits. An increase in content of total phenols is most probably a consequence of damaged epidermis in which the content of phenol was the highest; at the same time the epidermis become more permeable and make the extraction of phenol compounds easier in such condition. The content of total flavonoids and antioxidant activity did not follow the growth trend of phenol in methanol and ethylacetat extract; instead these values slightly decreased, while with acetone extract these values slightly rose.

The content of total phenols, flavonoids and antioxidant activity of black currant// in fresh and frozen condition is provided in Table 3.

Table 2. Content of total phenols (TP), flavonoids (TF) and of antioxidant activity (TAA) of extracts of fresh and frozen sour cherry in different solvents.

Solvent	Fresh fruits after picking			Fruits frozen at -20 °C for six months		
	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g
methanol	88,3	40,3	0,75	94,4	36,4	0,65
acetone	101,4	48,3	0,83	112,6	49,7	0,85
ethylacetate	87,6	41,4	0,77	90,4	38,8	0,68

TP total phenols expressed as mg equivalents of gallic acid in 100g of fresh or defrosted fruits; TF total flavonoids expressed as mg catechin equivalents in 100g of fresh or defrosted fruits; TAA antioxidant activity expressed as trolox equivalents.

Table 3. Content of total phenols (TP), flavonoids (TF) and of antioxidant activity (TAA) of extracts of fresh and frozen black currant in different solvents.

Solvent	Fresh fruits after picking			Fruits frozen at −20 °C for six months		
	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g	TP mgGAE/100g	TF mgCE/100g	TAA mmolTE/100g
methanol	124,4	58,6	0,93	148,4	56,4	0,85
acetone	123,6	53,7	0,85	140,6	59,7	0,95
ethylacetate	96,7	48,3	0,78	123,4	50,8	0,78

TP total phenols expressed as mg equivalents of gallic acid in 100g of fresh or defrosted fruits; TF total flavonoids expressed as mg catechin equivalents in 100 g of fresh or defrosted fruits; TAA antioxidant activity expressed as trolox equivalents.

The content of total phenols in the extract as well as the composition of extract largely depends on the method of extraction, type and polarity of solvent [13]. Presented results of the content of total phenols indicate that methanol proved as the most efficient solvent. The highest amount of flavonoid is also found in methanol extract, which is in accordance with the results given by other researchers too [14], while the antioxidant capacity is in positive correlation with phenol and flavonoid values. All extracts of frozen fruit have by 12 to 21.6% higher content of total phenol compared to freshly picked fruits. As with acetone and ethylacetate extract there has been an increase in the content of flavonoid too, only with methanol extract there was a decrease of that content. Antioxidant capacity followed the same increase and decrease of total flavonoids. In this study currant proved the highest content of total phenol which is in conformity with the results of other researchers [15]. High level of total phenols and of antioxidant activity of currant is a consequence of high content of vitamin C and of anthocyanin in them [16].

4. CONCLUSION

Fruits and fruit products exhibit strong biological effect which is primarily attributed to high content of phenolic compounds. In this study special attention was paid to the selection of a solvent for extraction of phenolic compounds since the extraction method and polarity of solvent are known to influence the amount of extracted secondary metabolites. Acetone proved to be the most efficient solvent for grapes and sour cherry, while the highest

content of total phenols from the black currant was obtained by extraction with methanol. The highest content of these compounds was found in black currant fruits, which may be accounted for by the fact that this fruit also has the highest content of anthocyanin. Fruits of currant had the highest antioxidant capacity, which is in conformity with the amount of phenolic compounds as well as some other compounds such as vitamin C which also exerts very pronounced antioxidant activity.

In all tested fruits stored in frozen condition there has been an increase of content of total phenols compared to their content in fresh fruits. This can be explained by easier extraction from epidermis which was previously degraded by ice crystals.

A decrease of content of total flavonoids in frozen fruits as well as a decrease of antioxidant activity may be explained by the fact that the plant material contains other substances too that foster their effect, but are thermo-unstable, so that with their decomposition the antioxidant effects decreases.

Grapes, sour cherry and black currant that are tested in this study, have lower values of content of total phenols and flavonoids compared to the results of many authors; however, the explanation may be sought in the fact that besides genetic predisposition, ecological factors have a significant influence on the content of phenolic matters (i.e. temperature, light and air humidity, degree of fruit maturity).

Presented data on the content of total phenolic components and flavonoids in methanol, acetone and ethylacetate extracts and of antioxidant activity of extracts shows that the obtained extracts represent a potential source of natural antioxidants.

As these are the fruits with high nutritive and antioxidant values, efforts should be made to promote their role in human diet.

Deep-freezing of the fruits at the temperature of −20 °C is the best way to preserve these fruits.

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ОДРЕЂИВАЊЕ СЕКУНДАРНИХ МЕТАБОЛИТА И ЊИХОВЕ АНТИОКСИДАТИВНЕ АКТИВНОСТИ У ВОЋУ

Сажетак: У раду је испитан утицај начина екстракције са различитим растварачима на садржај укупних фенола, флавоноида и на антиоксидативна својства екстраката грожђа, вишње и црне рибизле. Антиоксидативна активност екстраката на стабилни 1,1-дифенил-2-пикрил хидразил (DPPH) радикал одређена је спектофотометријски. Садржај укупних фенола је одређен Folin-Ciocalteu методом, а садржај укупних флавоноида спектофотометријским одређивањем примјеном $AlCl_3$. Праћене су и промјене вриједности ових параметара након замрзавања овог воћа.

У екстрактима је одређен висок садржај укупних фенола и флавоноида. Као најбоље средство за екстракцију грожђа и вишње показао се ацетон, док је највећи садржај укупних фенола код црне рибизле добијен екстракцијом метанолом. Садржај укупних фенола код воћа које је замрзавано се повећао али је дошло до пада укупних флавоноида и антиоксидацијске активности. Добијени резултати показују да се екстракти грожђа, вишње и црне рибизле могу употријебити као природни антиоксиданси.

Кључне ријечи: екстракција, феноли, флавоноиди, антиоксидативна активност.