Polyphenol contents and antioxidant activity

of five fresh fruit *Morus* spp. (*Moraceae*) extracts

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ABSTRACT: The aim of this research was determination of total phenolic, flavonoid and anthocyanin contents as well as measuring antioxidant activity in three types of mulberry (black, *Morus nigra L.*; red, *Morus rubra L.* and white, *Morus alba L.*) in five solvents (methanol, methanol-water (50/50, v/v%), ethanol, ethanol-water (50/50, v/v%) and water) grown in South East Serbia. The total phenolic content was determined using the Folin-Ciocalteu assay. The content of total flavonoids was measured spectrophotometrically using the aluminum chloride assay. The content of monomeric anthocyanins was measuring also spectrophotometrically using the pH differential method. Antioxidant assay was based on the measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorbance at 517 nm caused by the reaction of DPPH with the test sample. The results showed the highest total phenolic content in water extract of *Morus rubra L.*, the highest flavonoid content in methanol extract of *Morus nigra L.* and the highest anthocyanin content and antioxidant capacity in the DPPH assays showed the water extract of *Morus nigra L.*

INTRODUCTION

Morus, a genus of flowering plants in the family *Moraceae,* comprises 10–16 species of deciduous trees commonly known as mulberries. The most popular species of genus *Morus* are *Morus alba* L. (white mulberry), *Morus rubra* L. (red mulberry) and *Morus nigra* L. (black mulberry). Mulberry is found from temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere and they can grow in a wide range of climatic, topographical and soil conditions. These are widely spread throughout all regions from the tropics to the sub-arctic areas. Genus Morus is widespread in Asia, Europe, North and South America and Africa as well.

Mulberry fruit may be coloured white, red or black when they are ripe. Deep-coloured fruits are good sources of phenolics, including flavonoids, anthocyanins and carotenoids (1-4) and mulberries are rich in phenolics (5). Mulberry fruit has been used as a folk remedy to treat oral and dental diseases, diabetes, hypertension, arthritis and anemia (6). The main use of mulberry globally is as feed for the silk worm, but, depending on the location, it is also appreciated for its fruit (consumed fresh, in juice or as preserves), as a delicious vegetable (young leaves and stems), for its medicinal properties in infusions (mulberry leaf tea), for landscaping and as animal feed (7). Mulberry fruit can be used for making jam, jelly, pulp, fruit drink, fruit sauce and cake. Many desserts are made from the Persian mulberries along with sauces, pie-making, cakes and jelly, fruit tea (8-10).

The plant has high level of phenolic compounds, hence it has a very important role in the food industry. It is considered that the fresh fruit colour comes from anthocyanins present in the fruit. This has contributed to the positive effects of fruit on the people's health. The total content and the yield (percentage) of these compounds is dependent on geographic location and soil on which the mulberry tree grows. Accordingly, the results obtained in this study differ from the results of *Morus species* found in other countries.

With the aim of finding new sources of natural antioxidants, plants, fruits, vegetables and other plant materials that are known to possess antioxidant activity, have been investigated (11-14).

Many studies have investigated the contents of phenolics compounds in mulberry fruits (15-17). Several studies have previously reported that anthocyanins display significant antioxidant activity (18-19). There is growing interest for natural antioxidants, which prevent oxidation disorders in humans, rather than synthetic antioxidants, which are identified as carcinogens (20).

Despite previous research on this plant, there is no information in phenol compunds in fruit extracts of *Morus* spp. grown in Southeast Serbia.

MATERIAL AND METHODS

Preparation of the fresh fruit extracts

Plant material was collected in the South East Serbia in early July 2011. Fresh fruit maturity was estimated on the basis of the colour. Samples were stored in plastic bags and kept frozen until extraction. The frozen fresh fruit material homogenized using a blender. Black, red and white mulberry fresh fruits (10 g) was extracted with water, methanol-water (50/50, v/v%), methanol, ethanol-water (50/50, v/v%) and ethanol. All solvents were acidified with 1 ml conc. HCl. The extraction was performed with 100 ml of solvents using the ultrasonic bath for 30 minutes. The suspension was gravity filtered through a Buchner funnel and Whatman No. 1 filter paper. Extracts were stored in the fridge at 5°C until their analysis.

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), catechin and $AlCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid (Trolox) was purchased from Acros Organics (New Jersey, USA).

Folin- Ciocalteu's phenol reagent and sodium carbonate were purchased from Merck Chemical Suppliers (Darmstadt, Germany). Sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were purchased from the same producer. The other used chemicals including solvents were of analytical grade. An Agilent 8453 UV/Vis spectrophotometer was used for absorbance measurements and spectra recording, using an optical or quartz cuvettes of 1 cm optical path. The pH measurements were made with Hanna Instruments pH- meter equipped with glass electrode.

Determination of the total phenolic compounds

Total phenol contents of the extracts were determined by the modified Folin-Ciocalteu method (21). An aliquot of the extracts (1 ml) was mixed with 0.5 ml Folin-Ciocalteu reagent and 2 ml of sodium carbonate (20%). Absorbance was measured after 10 min incubation at room temperature at 760 nm. Total phenolic content was expressed as mg/100g gallic acid equivalent (GAE). The result of each one assay was obtained from three parallel determinations.

Determination of the total flavonoid content

Total flavonoid content was determined using a spectrophotmetric method based on formation of flavonoid complex with aluminum (22). Black, red and white mulberry extract (1 ml) was mixed with 3 ml deionized water and 0.3 ml $NaNO_2$. After standing at room temperature for 5 minutes, 3 ml $AlCl_3$ was added to the solution, followed by the addition of 2 ml of NaOH after another 5 minutes standing. The solution was than filled up to the line with deionized water in a 10 ml flask. The absorbance of the prepared solution was measured at 510 nm. Total flavonoid content was calculated as catechin (mgCE/100g) using the equation based on the calibration curve.

Determination of the total monomeric anthocyanins

The total monomeric anthocyanin content in the plant extracts was determined using the pH-differential method previously described (23). Anthocyanins demonstrate maximum of absorbance at 520 nm at pH 1.0. The coloured oxonium form of anthocyanin predominates at pH 1.0, and the colorless hemiketal form at pH 4.5. The pH-differential method is based on reaction producing oxonium forms. This allowed accurate and rapid measurement of total monomeric anthocyanins. Total monomeric anthocyanin pigment is expressed as mg of cyanidin-3-O-glucoside, by using molar absorptivity (ε) of 26900 and molecular weight of 449.2. For this method, 1 ml of the black, red and white mulberry extract, prepared by previously described procedure, was poured into two separate 10 ml volumetric flasks. Then, one was filled up to the line with solution of potassium chloride (KCl) (pH=1), and the second with sodium acetate (CH_3COON_a) (pH=4.5). The two diluted solutions were left to stand for 15 minutes at room temperature in dark. Finally, the absorbance of both samples was measured at λ_{max} 520 nm and 700 nm. Absorbances (A) of the investigated extracts were calculated by Eq 1.

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH4.5} \quad (1)$$

Content of the monomeric antocyanin pigment (MAP) was calculated by Eq 2.

$$MAP(mgl^{-1}) = (A \cdot MW \cdot DF \cdot 1000)/(\varepsilon \cdot l) \quad (2)$$

where A is absorbance were calculated by equation 1, ε is the molar absorptivity (26.900 $dm^3mol^{-1}cm^{-1}$), MW is the molecular weight (449.2gmol^{-1}), and DF is the dilution factor, *l* is the path length (1cm). The result, taken as the monomeric anthocyanin pigment (MAP), was expressed as mg of cyanidin-3-O-glucoside dm^{-3}.

Free radical scavenging activity

The free radical scavenging activity of the plant extracts was analyzed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (24-28). The antioxidant assay is based on the measurement of the loss of colour of DPPH solution by the change of absorbance at 517 nm caused by the reaction of DPPH with the tested sample. The reaction was monitored using UV-VIS spectrophotometer. Plant extracts 1 ml, 5 ml of freshly prepared DPPH in methanol and 4 ml of water were put into a cuvette at room temperature. After 30 minutes of incubation period at room temperature, the absorbance was read against a blank at 517 nm. All measurements were performed in triplicate at a final concentration. The ability of extracts to inhibit DPPH in percents (RSC %) was calculate from the decrease of absorbance according to the relationship (Eq 3.):

$$RSC\ (\%) = (1 - A_{sample}/A_{blank}) \cdot 100 \quad (3)$$

where A_{blank} is the absorbance of control($1 \cdot 10^{-4}$ mol dm^{-3} DPPH methanol solution), and A_{sample} is the absorbance of the test sample.

The final results were expressed as milligrames of Trolox equivalents (TE) per 100g of fresh sample (mgTE/100g).

Statistical analysis

The experimental results were expressed as mean value ± standard error of mean value of three replicates. In order to estimate statistically any significant differences among mean values, where it was applicable, the data were subjected to a one-way analysis of variance (ANOVA test), and differences among samples were determined by Duncan's Multiple Range test using the Statistical Analysis System (SAS, 1999) software (29).

RESULTS

The results show that the content of total phenols in the investigated extracts of *Morus nigra* L. was ranging from 45.45 mgGAE/100g to 134.42 mgGAE/100g for the methanol-water (50/50 v/v%) and methanol extract of fresh fruit, respectively. The content of total phenols of *Morus rubra* L. was found to be 56.40 to 320.78 mgGAE/100g for the methanol-water (50/50 v/v%) and ethanol-water (50/50 v/v%) extract, respectively. The highest content of phenols of *Morus alba* L. extract was in the ethanol extract (432.50 mgGAE/100g) and the lowest amount was identified in the methanol-water (50/50 v/v%) extract (70.87 mgGAE/100g) (Figure 1). Five analysed extracts gave different values for phenols,

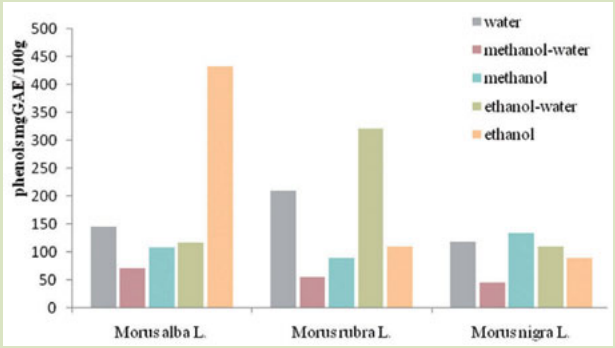

Figure 1. Total phenol contents in water, methanol-water (50/50 v/v%), methanol, ethanol-water (50/50 v/v%) and ethanol extracts of *Morus* species.

flavonoids and anthocyanins contents and different antioxidant activity as well. The difference in contents of phenolic compounds is a consequence of different solvent polarity and solubility of oxidant compounds in a given solvent.

Total flavonoid contents of the extracts of *Morus nigra* L. fresh fruit was ranging from 112.00 mgCE/100g (methanol-water (50/50 v/v%)) to 210.70 mgCE/ 100g (methanol extract), while in *Morus rubra* L. was ranging from 38.39 mgCE/100g (methanol-water (50/50 v/v%)) to 192.07 mgCE/100g fresh fruit (water extract), and in extracts of *Morus alba* L. was ranging from 29.00 (methanol-water (50/50 v/v%)) to 137.86 mgCE/100g (methanol extract) (Figure 2).

Anthocyanin contents of *Morus nigra* L. extracts was between 44.34 mg cyanidin 3-glucoside/100g for methanol-water (50/50 v/v%) and 128.68 mg cyanidin 3-O-glucoside/100g for ethanol-water (50/50 v/v%) extract. The extracts of *Morus rubra* L. showed anthocyanins

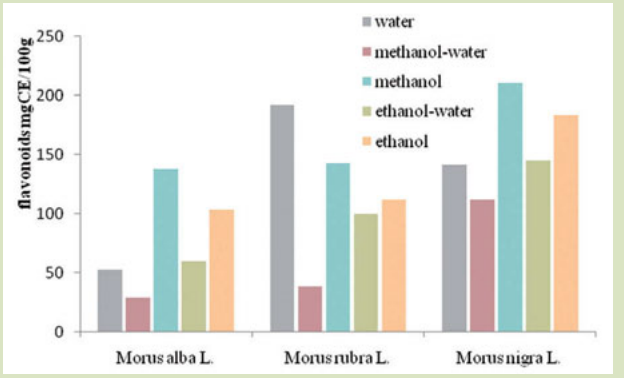

Figure 2. Total flavonoid contents in water, methanol-water (50/50 v/v%), methanol, ethanol-water (50/50 v/v%) and ethanol extracts of *Morus* species.

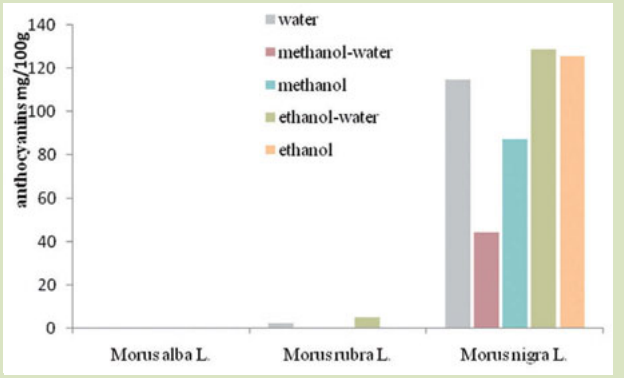

Figure 3. Total anthocyanin contents in water, methanol-water (50/50 v/v%), methanol, ethanol-water (50/50 v/v%) and ethanol extracts of *Morus* species.

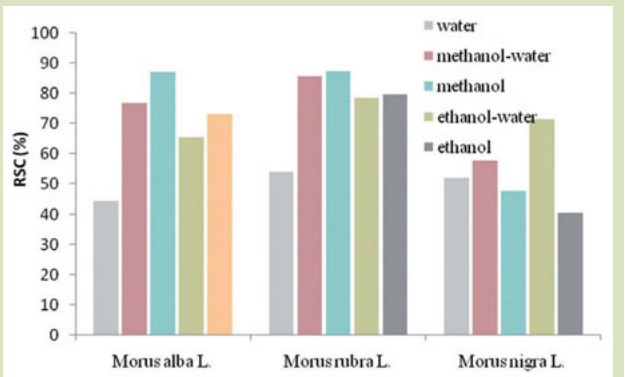

Figure 4. DPPH radical scavenging power of water, methanol-water (50/50 v/v%), methanol, ethanol-water (50/50 v/v%) and ethanol extracts of *Morus* species.

content only in water and ethanol-water (50/50 v/v%) extract (2.59 mg and 5.34 mg of cyanidin 3-glucoside/100g). The extracts of white mulberry did not show the anthocyanin content (Figure 3).

All the extracts exhibited good scavenging activity against DPPH radicals from 40.55% (*Morus nigra* L., ethanol extract) to 87.56% (*Morus rubra* L., methanol extract (Figure 4).

DISCUSSION

Previous studies examined the total content of phenols, flavonids and anthocyanins and antioxidant activity of *Morus* species from different countries and regions. For example, Ozgen *et al* found the phenol content (270 mgGAE/100g fruit of *Morus nigra* L. acetone extract and 160 mgGAE/100g fresh fruit of *Morus rubra* L. (6). Ercisli *et al* discovered significantly higher values of total phenol content in the plant of 1422 mgGAE per 100g fruit (30) of *Morus nigra* L. extract, 1035 mgGAE/100g for *Morus rubra* L. and 181 mgGAE/100g fresh fruit of *Morus alba* L. methanol extract. The phenol content of *Morus nigra* L. was high using the extraction with methanol in soxlet, 1943 mg-2237 mgGAE/100g fruit (15). *Morus alba* L. from Korea had 152 mg – 257 mgGAE/100g in ethanol extract (31). The ethanol extract of black mulberry from Korea gave 867 mgGAE/100g (32). *Morus nigra* L. from Pakistan had 661 mgGAE/100g and *Morus alba* L. showed 775 mgGAE/100g (33). *Morus alba* L. from Taiwan gave 1515 mgGAE/100g fresh fruit (5). *Morus rubra* L. grown in Brasil had 373 mgGAE/100g of fresh fruit (34) (Table 1).

Sample	Plant origin	Solvent	Total phenols content	References
Morus alba L.	Taiwan	Water	1515[a]	5
Morus alba L.	Turkey	Methanol	181[a]	30
Morus alba L.	Korea	Ethanol	2570[a]	31
Morus alba L.	Pakistan	Methanol	775[a]	33
Morus alba L.	Serbia	Ethanol/water	4.13[b]	36
Morus rubra L.	Turkey	Acetone	160[a]	6
Morus rubra L.	Turkey	Ethanol	169[a]	35
Morus rubra L.	Turkey	Methanol	1035[a]	30
Morus rubra L.	Brasil	Methanol	373[a]	34
Morus nigra L.	Turkey	Acetone	270[a]	6
Morus nigra L.	Turkey	Methanol	1943-2237[a]	15
Morus nigra L.	Turkey	Methanol	330-580[a]	17
Morus nigra L.	Turkey	Ethanol	215[a]	35
Morus nigra L.	Turkey	Methanol	1422[a]	30
Morus nigra L.	Korea	Ethanol	867[a]	32
Morus nigra L.	Pakistan	Methanol	775[a]	33
Morus nigra L.	Serbia	Ethanol/water	6.37[b]	36

Table 1. Comparison of levels of phenolic compounds from black, red and white mulberry grown in other regions.
[a]Total phenols (TP) expressed as gallic acid equivalents per 100g fresh fruit, bexpressed as mg of chlorogenic acid equivalents per gram of dry extracts.

Also, Kutlu *et al* used acidified methanol, acidified water and non-acidified methanol-water (70/30 v/v%) for the extraction of phenolic compounds in *Morus nigra* L. fruit. The highest content of phenolic compounds was found in non-acidified methanol-water (70/30 v/v%) (580 mg GAE/100g) extract, while water extract exhibited the lowest amount of phenols (330 mgGAE/100g). The methanol extract had 440 mgGAE/100g fresh fruit. The authors concluded that the increase of acidity does not increase the amount of extracted phenols (17). Ercisli *et al* analyzed the phenolic content using Folin Ciocalteu method from the black and red mulberry collected in Turkey (35). Their results indicated that the plant contained phenols 215 mgGAE/100g (*Morus nigra* L.) and 169 mgGAE/100g (*Morus rubra* L.) (Table 1), which was more than the contents of phenolics from a black and red mulberry tree

from a Southeast Serbia. Radojkovic *et al* have examined the white and black mulberry but they have been applied to different extraction and solvents (36). The differences in total phenol compounds have been found to be dependent on the extraction medium used and polarity of the organic solvents used for the extraction. The extraction duration, method and the plant material condition attribute to the content of phenols.

In previous studies, flavonoids content ranged from 0.56 mgCE to 6.54 mgCE/100g (31) of *Morus alba* L. which is found to be much less compared to our findings.

The anthocyanins analysis of black mulberry from Turkey had 720 mg Cy-3-O-glucoside per 100 g fruit and 109 mg Cy-3-O-glucoside per 100 g fruit for *Morus rubra* L. (35). Our analysis showed that black mulberry contains less value of anthocyanins ranged from 74.72 to 114.83 mg Cy-3-O glucoside per 100 g of fruit for black mulberry and from 1.77 to 5.28 mg Cy-3-O glucoside per 100 g of fruit for red mulberry. Black mulberry from Korea contained 571 mg Cy-3-O-glucoside per 100g dry fruit (6). The white mulberry from Serbia (36) does not contain anthocyanins. Literature review by authors from different countries indicates that all of compounds: total phenols, flavonoids and anthocyanins varies depending on the geographic and climatic factors. Methanol extract of black mulberry from Turkey had an antioxidant activity of 95 %, aqueous 80 % and methanol-water (70/30, v/v%) 85 % (17). Other studies have shown that the activity, was expressed as Trolox equivalent, was 748 mgTE/100g and 442 mgTE/100g for black and red mulberry which was considerably higher than our results (35).

In the world, mulberry is generally used as forage in animal production. The full-bodied flavour of this fruit is a good balance of sweetness and tartness with nutrient elements of vital importance for human metabolism. If these fruits are industrially exploited for various commercially valuable products, mulberry can become an important crop throughout the world. Mulberry fruit can be used for making jam, jelly, pulp, fruit drink, fruit sauce and cake. Many desserts are made from the Persian mulberries along with sauces, pie-making, cakes and jelly (8), fruit tea. In Chinese markets, mulberry is often provided in the form of a paste called sangshengao. This paste is mixed in hot water to make a tea to improve the liver and kidney and sharpen the hearing and brighten the eyes (37). Mulberry fruits can be dried and stored as a powder. About 10 g of dried fruits provides about 100 mg of anthocyanins. The fruit powder has an anti-aging effect on cells because it combats free radical damage. Fruit powder promotes maintain normal level of cholesterol and controls carbohydrate digestion in the human body (9). Over-ripened and sour fruits can be converted into mulberry wine. The wine has a sweet and sour taste. A glass of mulberry wine a day helps get rid of impurities and coprostasis (faecal residue in the intestines) in the body which can help make the body slim. Mulberry fruit wine is very popular as a ladies drink in Europe (38). Mulberry fruits are rich in anthocyanins and deserve to be exploited for the industrial production of natural colour to be used in the food industry.

CONCLUSIONS

In this study, we compared phenolic, anthocyanin, flavonoids content and antioxidant activity of different extracts of *Morus nigra* L., *Morus rubra* L. and *Morus alba* L. fresh fruit from Serbia. The high phenolic content and high antioxidant activity of black mulberry from Southeast Serbia underline the nutritive and phytomedicinal potentials of the fruit. Further studies are, however, required before the fruit extract can be exploited in the production of health foods and as an antioxidant carrier in the food and pharmaceutical industries. This study suggested a use of mulberry as potential healthy foods, or important antioxidant carrier in food and pharmaceutical industries.

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REFERENCES

1. R. Zadernowski, M. Naczk, Nesterowicz, J. *J Agric Food Chem.,* **53**, pp. 2118-2124 (2005).
2. A. Sass-Kiss, J. Kiss J. et al., *Food Res Inter.,* **38**, pp. 1023-1029 (2005).
3. J.Y. Quian, D. Liu, A.G. Huang. *Food Chem*., **87**, pp. 283-288 (2004).
4. A. Trappey, A. Bawadi et al., *Food Chem*., **91**, pp. 665-671 (2005).
5. J.Y. Lin, C.Y. Tang. *Food Chem.,* **101**, pp. 140-147 (2007).
6. M. Ozgen, S. Serce et al., *Sci Hortic*., **119**, pp. 275-279 (2009).
7. M. Imran, H. Khan et al., *J Zhejiang Univ-Sci B*, **11**, pp. 973-980 (2010).
8. www.tytyga.com (2009).
9. L.K. Liu, F.P. Chou et al., *J Agric Food Chem.*, **57**, pp. 7605-7611 (2009).
10. R.E. Wrolastad. The posible health benefits of anthocyanin pigments and polyphenolic, Linus Pauling Institute, Oregon state University. (2001).
11. G. Miliauskas, P.R. Venskutonis, T.A. Beek, *Food Chem*., **85**, pp. 231-237 (2004).
12. K.H. Sim, H.Y.Sil, *Int J Food Sci Tech*., **43**, pp. 1813-1823 (2008).
13. L.C. Ryan, M.G. Mestrallet et al., *Int J Food Sci Tech.*, **43**, pp. 193-199 (2008).
14. R. Olmedo, V. Nepote et al., *Int J Food Sci Tech.,* **43**, pp. 1935-1944 (2008).
15. O. Ercsli, E. Orhan, *Sci Hortic*., **116**, pp. 41-46 (2008).
16. D. Gerasopoulos, G. Stavroulakis, *J Sci Food Agric*., **73**, pp. 261-264 (1997).
17. T. Kutlu, G. Durmaz et al., *Turk J Biol.,* **35**, pp. 103-110 (2011).
18. F.C. Stinzing, A.S. Stinzing et al., *J Agric Food Chem.*, **50**, pp. 6172-6181 (2002).
19. H. Wang, G. Cao, R.L. Prior., *J Agric Food Chem.*, **45,** pp. 304-309 (1997).
20. N. Ito, S. Fukushima et al., *J Natl Cancer Inst.*, **70**, pp. 343-347 (1983).
21. V.L. Singleton, J.A. Rossi, *Am J Enol Viticult*, **16**, pp. 144-158 (1965).
22. E.Z. Ordom, J.D. Gomez et al., *Food Chem*., **97**, pp. 452-458 (2006).
23. M. Guisti, R. Wrolstad, Current protocols in food analytical chemistry. New York: John Wiley and Sons, (2003).
24. B. Fuhrman, N. Volkova et al., *J Agric Food Chem.*, **49,** pp. 3164-3168 (2001).
25. J. Lachman, M. Šulc, M. Schilla, *Food Chem*., **103, p.** 802-807 (2007).
26. J. Sanchez-Moreno, J.A. Larrauri, F. Saura-Calixto, *J Agric Food Chem*., **79**, pp. 1301-1304 (1999).
27. A. Turkoglu, M.E. Duru et al., *Food Chem*., **101,** pp. 267-273 (2007).
28. D. Villano, M.S. Fernandez-Pachon et al., *Food Chem*., **95,** pp. 394-404 (2006).
29. Statistical Analysis and Reporting System, ser Guide, version 1.0, 1MB, (1999).
30. S. Ercisli, E. Orhan, *Food Chem.*, **103,** pp. 1380-1384 (2007).
31. S.H. Bae, H.J. Suh, *LWT - Food Sci & Techol*., **40**, pp. 955-962 (2007).
32. W. Chun, X. Li et al., *Afr. J. Biotechnol*. **10,** pp. 16175-16180 (2011).
33. A.A. Memon, N. Memon et al., *Pol. J. Food Nutr. Sci,* **60,** pp. 25-32 (2010).
34. N.M.A. Hassimotto, M.I. Genovese, F.M. Lajolo, *Food Sci Tech Int.,* **13,** pp. 17-25 (2007).
35. S. Ercisli, M. Tosun et al., *Food Technol. Biotechnol.*, **48**, pp. 102-106 (2010).
36. M.M. Radojkovic, Z.P. Zekovic et al., *Hem. Ind*. doi:10.2298/HEMIND111111002R (2012)
37. www.itmonline.com (2009)
38. www.eHow.com