ANTIOXIDANT ACTIVITY OF WHITE GRAPE SEED EXTRACTS ON DPPH RADICALS

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Composition and antioxidant activity of grape seed extract (GSE) obtained from red grape varieties are very well documented, in contrast to the white varieties. This paper presents the results of polyphenols content of ethyl acetate extract of grape seeds, obtained from two white grape varieties, Italian Riesling and Župljanka, and their antioxidant activity on the stable DPPH radical. The influence of the addition of GSE to raspberry juice on the DPPH radical was also examined. Content of total polyphenols in GSEs ranged between 81.6 and 82.8% (w/w), and the content of flavan-3-ols between 66.2 and 91.0% (w/w). HPLC results showed that the most abundant components in the extract were (+)-catechin and (-)-epicatechin for both grape varieties. All tested GSEs exhibited good antioxidant activity. IC₅₀ values for the GSEs of Italian Riesling and Župljanka were 0.79 and 0.95 mg sample/mg DPPH radical, respectively. Since the GSE of Italian Riesling possessed stronger antioxidant activity, it was used for further experiments. The IC₅₀ value for raspberry juice was 4.18 mg raspberry juice/mg DPPH. The raspberry juice with addition of 0.60 µg/mL of GSE showed antioxidant activity of 39.2%. The same juice with the threefold concentration of vitamin C (1.81 µg/ml) exhibited similar antioxidant activity (33.9%). Antioxidant activity of the same amount of juice without added antioxidants was lower (15.7%). The results showed that the GSE of white varieties could be considered as a good functional food ingredient.

KEY WORDS: Grape seed extract, white grape variety, antioxidant activity, polyphenolics

INTRODUCTION

The use of synthetic antioxidants in the food has been under scrutiny for toxicological reasons, and therefore the interest in the natural antioxidants has steadily been increasing (1-3). The antioxidant and radical scavenging activities of a large number of polyphenolic compounds isolated from plants have been studied (4-8). Green tea, grape seeds and skin
are considered as the remarkable rich sources of polyphenolics (6). Composition and antioxidant activity of grape seed extract (GSE) obtained from red grape varieties are very well documented, in contrast to the white varieties (9, 10). The GSE rich in polyphenols, is a mixture of proanthocyanidines: monomers, oligomers and polymers of flavan-3-ols ((+)-catechin, (-)-epicatechin, (-)-epicatechin-O-gallate, and (-)-epigallocatechin) linked with C4-C8 or C4-C6 bonds. The biological, pharmacological and medicinal properties of the bioflavonoids and proanthocyanidins have been extensively reviewed. Besides the free radical scavenging and antioxidant activity, proanthocyanidins exhibit vasodilatory, anticarcinogenic, anti-allergic, anti-inflammatory, antibacterial, cardioprotective, immune-stimulating, anti-viral and estrogenic activities, as well as they act as inhibitors of the enzymes phospholipase A2, cyclooxygenase and lipooxygenase (9).

The aim of this work was to determine the polyphenolic composition and antioxidant activity on DPPH radical of GSEs obtained from two white grape varieties, Italian Riesling and Župljanka. Also, the influence of the addition of GSE to a raspberry juice on the DPPH radical was examined.

**EXPERIMENTAL**

**Chemicals and Samples**

All solvents used for the extraction and spectrophotometric determination were of analytic grade. Methanol and ethanol were purchased from Lachema (Neratovice, Czech Republic) and ethyl acetate from Kemika (Zagreb, Croatia). Acetonitrile and methanol, LiChrosolv, gradient grade for chromatography and vanillin were obtained from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazil stable radical (DPPH), gallic acids, (+)-catechin and (±)-epicatechin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Folin-Ciocalteau reagent was obtained from Fluka (St. Gallen, Switzerland).

The sample, pomace of *Vitis vinifera* cultivars, Italian Riesling and Župljanka, were collected at the “Navip” winery. The grapes used for processing were harvested at optimum technological maturity, as judged by the indices of sugar and acid contents, established by the laboratory in the “Navip” winery. The average sugar content in grapes was 17.0%, and the acid content 6.4%. After pressing, the pomace was sampled for experiments. The seeds were separated from other pomace components (skin and stems), dried at room temperature, and stored at 24°C prior to extraction.

A raspberry sample was purchased from a local market. Juice was separated from the pulp, and the freshly obtained juice was two times diluted with water and used in the antioxidant assay procedure.

**Extraction procedure**

The extraction was carried out according to the method described by Pekić *et al.* (11). 100 g of grape seeds was extracted with 400 mL of 90% ethyl acetate in a sealed bottle at room temperature for 48 h, with occasional mixing. The obtained extract was filtrated, and the solvent was removed by evaporation under the reduced pressure to the volume of approximately 20 mL, at maximum temperature 40°C. The concentrated ethyl acetate solution was mixed with a five-fold volume of chloroform and the formed precipitate was
separated by filtration through a nutsch filter B-4. The precipitate was washed with chloroform, dried in a vacuum desiccator and the yield of obtained GSE was calculated.

**Total phenolics and flavan-3-ols in GSE**

The amount of the total soluble polyphenols in the GSE was determined spectrophotometrically according to the Folin-Ciocalteau method (12). Gallic acid was employed as a calibration standard and the results were expressed as gallic acid equivalents in grams per 1 kg of dry seed weight. The amount of total flavan-3-ols was assayed spectrophotometrically by the vanillin method using (+)-catechin as a standard (13,14). The values were expressed as catechin equivalents in grams per 1 kg of dry seed weight. All analyses were performed in triplicate and the results were averaged.

**HPLC Analysis of GSE**

HPLC analyses were conducted on a Hewlett-Packard Liquid Chromatograph HP 1090 equipped with Diode Array Detector (DAD). A reversed-phase column (Zorbax SB-C18, 5 µm, 3.0 x 250 mm i.d.), protected by guard column (Zorbax SB-C18, 5 µm, 4.6 x 12.5 mm i.d., Agilent, USA) was used throughout this research. The detection was performed at 277 nm and the absorption spectra of the compounds were recorded between 210 and 400 nm. The solvent gradient was formed by varying the proportion of the solvent A (1% acetic acid in water, v/v) to the solvent B (acetonitrile) (15). The solvent linear gradient elution programme was as follows: 0-20 min, 95-87% A; 20-30 min, 87% A; 30-46 min, 87-78% A; 46-55 min, 78-10% A; 55-65 min, 10% A. The column was equilibrated to the initial conditions, 95% A, 10 min. The flow rate was set at 0.300 mL/min. The column was operated at room temperature (22°C). The sample injection volume was 10 µL, and the injection was performed manually. The GSE was dissolved in 10% (v/v) methanol in water and the obtained concentration was 1.0 mg/mL. All solutions were filtered through 0.45 µm pore size nylon filters (Rotilabo–Spritzen-filter 13 mm, Roth, Karlsruhe, Germany) before injecting them into the HPLC system. Phenolic components in a sample extract were identified by matching the retention time and their spectral characteristics against those of the standards. The purity of the peaks was determined to ensure the identification. The external standard method was a technique used for quantification. For each component, (gallic acid, (+)-catechin, and (±)-epicatechin) a stock solution was made from the commercial standards dissolved in 10% (v/v) methanol in water to obtain the concentration of 1.0 mg/mL. The diluted stock solutions were used for calibration. The final concentrations were in the range of 0.005-0.200 mg/mL. The peak areas from the chromatograms were plotted against the known concentrations of the standards. The equations generated via linear regression were used to establish the concentrations of the phenolic compounds in the extracts. When reference compounds were not available, the calibration of (+)-catechin was used. The results were presented as mean values with the standard deviations (SD).

**Spectrophotometric assay for DPPH radical**

In a test tube containing 3 mL of methanolic GSE solution, prepared at five different concentrations ranged between 0.1 and 3.5 µg/mL, 1 mL of c(DPPH radical) = 90 µmol/L
(dissolved in 95% methanol in water, v/v) was added (test sample). The blank was prepared by adding 1 mL of DPPH radical solution to 3 mL of 95% methanol. The reaction mixture was allowed to stay in the dark at room temperature for 60 minutes.

The absorbance was read at 515 nm using 95% methanolic solution as a reference solution (16). The percentage of the remaining DPPH radical (%DPPH<sub>rem</sub>) and antioxidant activity (%AA) were calculated for each concentration of the GSE from the obtained absorbance for the sample (A<sub>s</sub>) and the blank (A<sub>0</sub>) using the following equations:

\[
\%DPPH_{\text{rem}} = \left( \frac{A_s}{A_0} \right) \cdot 100
\]

\[
\%AA = 100 - \%DPPH_{\text{rem}}
\]

The values of the calculated IC<sub>50</sub> were expressed as mg GSE/mg of DPPH radical. The IC<sub>50</sub> value is the concentration of antioxidant, required for 50% scavenging of DPPH radical in the specified time period.

Determination of DPPH scavenging activity of raspberry juice, vitamin C, and the juice with the addition of 0.60 µg/mL GSE or 1.81 µg/mL vitamin C, differed from the procedure given for GSE only in concentration range. Procedure for the scavenging activity of raspberry juice was as follows: 2 mL of raspberry juice, two times diluted with water, was further diluted to 50 mL with methanol, in order to prepare the stock solution. The final concentrations used for the assay were in the range of 0.05-0.50 mg/mL, which corresponds to 0.5-5.0 mL of fresh raspberry juice. Solution of vitamin C was prepared at five different concentrations ranging from 0.20 to 2.00 µg/mL.

**RESULTS AND DISCUSSION**

Proanthocyanidins from the grape seeds are extracted with the methanol, ethanol, acetone and ethyl acetate, or with their mixtures for analytical and preparative purposes (17). The differences in the GSE yields could be observed due to the extraction method used. The yield of the GSE extract obtained with ethyl acetate for the Italian Riesling was 6.03 ± 0.53 g/kg of dry seeds, and it was higher than the yields (3.6-4.7 g/kg of dry seeds) reported by Pekić et al. (11) for the same grape variety. These differences could be attributed to the variations due to the seasons within the grape variety. Additionally, genetic potential of the individual species for the polyphenol biosynthesis and maturation stage may affect polyphenol content in seeds, along with the variations from season to season (18-20). The yield of the GSE extract obtained with ethyl acetate for the Župljanka was 7.01 ± 0.75 g/kg of dry seeds.

In this paper, the total soluble polyphenols of the obtained GSE is expressed as gallic acid equivalents and for the Italian Riesling it was 4.92±0.30 g/kg of dry seeds, as regards 81.6% (w/w GSE). Content of total soluble polyphenols for the Župljanka was 5.80±0.28 g/kg of dry seeds, as regards 82.8% (w/w GSE). Comparing the yields obtained from the methanolic extracts, 1.84-4.07 g/kg of dry seeds, reported by Revilla et al. (21) for the white grape varieties, the yields were similar to or higher than those obtained in this research. The content of flavan-3-ols in GSE was 5.83±0.20 g/kg of dry seeds, as regards 91.0% (w/w GSE) for the Italian Riesling, and 4.64±0.20 g/kg of dry seeds, as regards
66.2% (w/w GSE) for the Župljanka. It may be concluded that the GSE of Župljanka contains higher amounts of tanins than the GSE of Italian Riesling, due to the fact that despite of the higher yield and total polyphenolics content of Župljanka the content of flavan-3-ols was lower. The obtained results are in accordance with the results of Nakamura et al. (17). According to their results, the differences in the sensitivity to the flavan-3-ols assay were observed, higher reactivity was observed in procyanidins which are highly polymerized and lower reactivity was observed in catechins which are highly esterified by gallic acid. In addition, epicatechin was more reactive than catechin.

![HPLC chromatogram of a GSE recorded at 277 nm. Peak assignment (tR, min): 1. gallic acid (9.3); 2. procyanidin-B3 (20.6); 3. procyanidin-B1 (22.4); 4. (+)-catechin (25.0); 5. procyanidin-B4 (27.5); 6. procyanidin-B2 (29.1); 7. (−)-epicatechin (33.4); 8. procyanidin-C1 (39.5); 9. dimer gallate (42.1); and 10. Procyanidin-B5 (49.9)](image-url)

A HPLC chromatogram of GSE is shown in Fig 1. The separation of the phenolic compounds in GSE was achieved within 60 min. Under the described chromatographic conditions, the components were eluted in the following order: gallic acid, procyanidin-B3, procyanidin-B1, (+)-catechin, procyanidin-B4, procyanidin-B2, (−)-epicatechin, procyanidin-C1, dimer gallate, and procyanidin-B5. The content of the total phenolic compounds determined by HPLC in GSE was 150 ± 2.10 mg/g GSE and 114 ± 2.90 mg/g GSE, for the Italian Riesling and Župljanka, respectively. A dominant compound was (+)-Catechin, with amount of 32.2 ± 1.30%, followed by (−)-epicatechin, 22.3 ± 0.72% for the Italian Riesling, and 30.6 ± 1.13%, followed by (−)-epicatechin, 26.8 ± 0.93% for the Župljanka. The content of phenolic compounds in the ethyl acetate extracts reported by Guendez et al. (18) was on average three times lower determined by HPLC than the one obtained by the Folin-Ciocalteau assay. These results are in agreement with the results obtained in this research. It could be assumed that lower results of polyphenolics content obtained by HPLC determination are due to the fact that only monomers, dimers
and trimers of procyanidins could be determined, while the obtained higher results for spectrophotometric assay are owing to the presence of highly polymerized procyanidins and other substances in extract.

Antioxidant activity of GSE is assessed by scavenging of the stable DPPH radical. With increasing concentrations of GSE, antioxidant activity on investigated free radical increased. The IC$_{50}$ for the GSEs of Italian Riesling and Župljanka were 0.79 and 0.95 mg sample/mg DPPH radical, respectively, and it is in the same range as the values obtained by Bakkalbaşi et al (22) for the GSEs of the white grape varieties using acetone for the extraction (0.52–0.82 mg sample/mg DPPH radical). The antioxidant activities of different concentrations of GSEs of Italian Riesling and Župljanka are presented on Fig. 2. Since the better antioxidant activity was obtained for the Italian Rieslings GSE, the extract of Italian Riesling was used for the further investigation.

![Fig. 2. The antioxidant activities of different concentrations of GSEs of Italian Riesling and Župljanka on DPPH radical](image)

The IC$_{50}$ values for the raspberry juice and vitamin C solution were 4.18 and 1.88 mg/mg DPPH, respectively. The raspberry juice with addition of 0.60 µg/mL of GSE showed the antioxidant activity of 39.2%. The same juice with the threefold concentration of vitamin C (1.81 µg/ml) exhibited similar activity (33.9%) on DPPH radical. Antioxidant activity of the same amount of juice without any addition of antioxidants was 15.7%.

**CONCLUSION**

The high flavan-3-ols content and the antioxidant activity on stable DPPH radical of the GSEs, obtained from white grape varieties, Italian Riesling and Župljanka, were determined. GSE exerts better antioxidant activity on DPPH radical in comparison with juice containing threefold concentration of vitamin C and juice without the addition of antioxidant. Antioxidant activity of GSE is further confirmed through the addition of GSE to
fresh raspberry juice, which resulted in stronger scavenging activity of DPPH radical than the activity of pure juice. Tested white grape varieties could be used as a source for producing GSE, like red varieties, which are commercially used for industrial production of GSE as a dietary supplement and a natural food additive.

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REFERENCES


АНТИОКСИДАТИВНА АКТИВНОСТ ЕКСТРАКАТА СЕМЕНА БЕЛОГ ГРОЖЂА НА DPPH РАДИКАЛЕ

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Састав и антиоксидативне особине екстраката семена из сорти црног грожђа су детаљно описане у литератури, док су екстракти семена сорти белог грожђа мање испитиване. У раду су приказани резултати одређивања садржаја полифенолних јединиња и антиоксидативне активности етилацетатног екстракта семена две сорте белог грожђа, Италијански ризлинг и Жупљанка, на DPPH радикал. Утврђен је утицај додатка екстракта, као антиоксиданта, у сок од малине на исту радикалску врсту. Садржај укупних полифенолних јединиња у екстрактима износио је од 81,6 до 82,8 % (w/w), а садржај флаван-3-ола је био између 66,2 и 91,0 % (w/w). Садржај најзаступљенијих компонената, (+)-катехина (32,17 ± 1,30%) и (-)-епикатехина (22,30 ± 0,72%), утврђени су HPLC методом. Сви испитивани екстракти показали су добру антиоксидативну активност. Вредност IC50 за екстракт семена грожђа сорте Италијански ризлинг износила је 0,79, а за екстракт сорте Жупљанка 0,95 mg узорка/mg DPPH. Пошто је екстракт семена грожђа сорте Италијански ризлинг по-
казао бољу антиоксидативну активност, он је коришћен за даља испитивања. IC50 вредност за сок од малине износила је 4,18 mg узорка/mg DPPH. Сок од малине са додатком екстракта од 0,60 µg/ml показао је антиоксидативну активност од 39,2%. Сличну антиоксидативну активност (33,9%) имао је и сок од малине са додатком витамина Ц у три пута већој концентрацији (1,81 µg/ml). Антиоксидативна активност исте количине сока без додатка антиоксиданата је била знатно нижа и износила је 15,7%. Наведени резултати испитивања указују на могућност коришћења екстракта семена сорти белог грожђа као доброг функционалног додатка.

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