Antioxidant and Antimicrobial Activities of Beet Root Pomace Extracts

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Abstract

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We described the *in vitro* antioxidant and antimicrobial activities of ethanol, acetone, and water extracts of beet root pomace. Total contents of phenolics (316.30–564.50 mg GAE/g of dry extract), flavonoids (316.30–564.50 mg RE/g of dry extract), betacyanins (18.78–24.18 mg/g of dry extract), and betaxanthins (11.19–22.90 mg/g of dry extract) after solid-phase extraction were determined spectrophotometrically. The antioxidant activity was determined by measuring the reducing power and DPPH scavenging activity by spectrometric metod, and hydroxyl and superoxide anion radical scavenging activity by ESR spectroscopy. In general, the reducing power of all the beet root pomace extracts increased with increasing concentrations. The DPPH-free radical scavenging activity of the extracts, expressed as EC_{50}, ranged from 0.133 mg/ml to 0.275 mg/ml. Significant correlation was observed between all phytochemical components and scavenging activity. 0.5 mg/ml of ethanol extract completely eliminated hydroxyl radical, which had been generated in Fenton system, while the same concentration of this extract scavenged 75% of superoxide anion radicals. In antibacterial tests, *Staphylococcus aureus* and *Bacillus cereus* showed higher susceptibility than *Escherichia coli* and *Pseudomonas aeruginosa.*

Keywords: *Beta vulgaris* L.; phenolic compounds; bacterial culture; betalains; radical scavengig activity; electron spin resonance

The association between the diet rich in fruits and vegetables and a decreased risk of cardiovascular diseases and certain forms of cancer is supported by considerable epidemiological evidence (Ness & Powles 1999; Riboli & Norat 2003). Different studies have shown that free radicals present in the human organism cause oxidative damage to various molecules, such as lipids, proteins, and nucleic acids, and are thus involved in the initiation phase of the degenerative diseases. Phenolic and other phytochemical antioxidants found in fruits and vegetables are capable of neutralising free radicals and may play a major role in the prevention of certain diseases (Kaur & Kapoor 2001).

Beet root (*Beta vulgaris* L. ssp. *vulgaris*, Chenopodiaceae) ranks among the 10 most powerful vegetables with respect to its antioxidant capacity ascribed to a total phenolic content of 50–60 μmol/g dry weight (Vinson *et al.* 1998; Kähkönen *et al.* 1999). Beet root is a potential source of valuable water-soluble nitrogenous pigments, called betalains, which comprise two main groups, the red betacyanins and the yellow betaxanthins. They are free radical scavengers and prevent active oxygen-induced and free radical-mediated oxidation of biological molecules (Pedreno & Escribano 2001). Betalains have been extensively used in the modern food industry. They are one of the most

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important natural colorants and are also one of the earliest natural colorants developed for the use in food systems (Francis 1999; Azeredo 2009). A more recent investigation showed that total phenolics content decreases in the order peel (50%), crown (37%), and flesh (13%). The peel also carries the main portion of betalains with up to 54%, their content being lower in crown (32%) and flesh (14%) (Kujala *et al*. 2000). Whereas the coloured fraction consists of betacyanins and betaxanthins, the phenolic portion of the peel shows L-tryptophane, *p*-coumaric and ferulic acids, as well as cyclodopa glucoside derivatives (Kujala *et al*. 2001). Also, beet root contains a significant amount of phenolic acids: ferulic, protocatechuic, vanillic, *p*-coumaric, *p*-hydroxybenxoi, and syringic acids. The high content of folic acid amounting to 15.8 mg/g dry matter is another nutritional feature of the beets (Wang & Goldman 1997).

On the industrial scale, juices are produced by pressing mashed fruits and vegetables, with or without the application of pectolytic enzyme preparations, using different equipment. However, most of the secondary plant metabolites and dietary fiber compounds are not transferred into the liquid phase during the dejuicing process and remain in the pomace after pressing (Will 2000). Though still rich in betalains and phenols, the beet root pomace from the juice industry (15–30%) is disposed of as feed and manure. Thus new aspects, concerning the use of these wastes as by-products for further exploitation, are gaining an increasing interest because these are high-value products and their recovery may be economically attractive (Schieber 2001).

In the food industry, synthetic antioxidants, such as butylated hydroxyanizole (BHA) and butylated hydroxytoluene (BHT), have long been widely used as antioxidant additives to preserve and stabilise the freshness, nutritive value, flavour and colour of foods, and animal feed products. However, at least one study has revealed that BHT could be toxic, especially at high doses (Schilderman *et al*. 1995). Nowadays, there is an increasing interest in the substitution of synthetic food antioxidants by natural ones. The antioxidant compounds from waste products of food industry could be used for protecting the oxidative damage in living systems by scavenging oxygen free radicals, and also for increasing the stability of foods by preventing lipid peroxidation (Makris *et al*. 2007). Special attention is focussed on their extraction from inexpensive or residual sources coming from agricultural industries. The objectives of this study were: (*i*) to examine the phenolic and betalain compositions of beet root pomace extracts using spectrophotometrical determination of total phenolics, flavonoids, and betalains, (*ii*) to determine the antioxidant activity of pomace extracts on stable 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), reactive hydroxyl ($^{\bullet}$OH), and superoxide anion ($O_2^{\bullet-}$) free radicals and the reducing power of the extracts obtained, (iii) to establish correlations between the phenolic and belalain compositions and antioxidant activity (*iv*) to determine the antibacterial activity.

MATERIAL AND METHODS

Chemicals. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO), KO_2 per crown ether, Folin-Ciocalteu reagent, BHA, chlorogenic acid and rutin were purchased from Sigma Chemical Co. (St. Louis, USA). These chemicals were of analytical reagent grade. Other chemicals and solvents used were of the highest analytical grade and were obtained from Zorka, Šabac (Serbia).

Plant material. Beet root pomace were obtained from the factory for fruit and vegetable processing Zdravo organic, Selenca, Serbia.

Bacterial cultures. *Escherichia coli* (ATCC 10526), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 11632), and *Bacillus cereus* (ATCC 10876) microorganism strains were employed for the determination of antimicrobial activity. The cultures of the test bacteria were grown 20–24 h in Müller-Hinton agar (Torlak, Belgrade, Serbia) at 37°C and then transferred to Müller-Hinton broth. The inocula were prepared by adjusting the turbidity of the medium to match 5×10^6 CFU/ml.

The antibiotic chloramphenicol used as reference standard was obtained from Sigma-Aldrich (Steinheim, Germany).

Bacteria were obtained from the stock cultures of Microbiology Laboratory, Faculty of Technology, University of Novi Sad.

Extraction procedure. The samples of beet root pomace (40 g) were extracted at room temperature for 2 × 24 hours. The extraction was performed with different solvents: 80% ethanol aqueous solution containing 0.5% acetic acid (E1), 80% acetone

aqueous solution containing 0.5% acetic acid (E2), and 0.5% acetic acid in water (E3). The extracts obtained were combined and evaporated to dryness under reduced pressure. The yields of extracts were: m_{E1} = 3.32 g; m_{E2} = 3.09 g, and m_{E3} = 3.55 g.

Extract purification. A solid-phase extraction (SPE) with a vacuum manifold processor (system spe-12G; J.T. Baker, Großgerau, Germany) with CHROMABOND C_{18} column (1000 mg; J.T. Baker, Phillipsburg, USA) was used for the extract purification in order to remove the organic acids, residual sugars, amino acids, proteins, and other hydrophilic compounds (Rigo *et al*. 2000). The CHROMABOND C_{18} column was preconditioned by passing 8 ml of methanol and 20 ml of 5mM H_2SO_4. Beet root pomace extract was dissolved in 10 ml of 0.5M H_2SO_4. The extract solution was loaded onto the preconditioned column. The column was washed with 8 ml of 5mM H_2SO_4. The purifed extract was eluted with 8 ml of methanol and 20 ml of distiled water. The purified beet root extract was evapored to dryness under reduced pressure. The obtained weights of SPE-purified extracts were: m_{pE1} = 0.130 g; m_{pE2} = 0.085 g and m_{pE3} = 0.128 g.

Total phenolic content. The amount of total soluble phenolics in the extracts was determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton *et al*. 1999). The reaction mixture was prepared by mixing 0.1 ml of water solution (concentration 1 mg/ml) of the extract, 7.9 ml of distilled water, 0.5 ml of Folin-Ciocalteu's reagent and 1.5 ml of 20% sodium carbonate. After 2 h, the absorbance at 750 nm (UV-1800 spectrophotometer, Shimadzu, Kyoto, Japan) was read against control that had been prepared in a similar manner, by replacing the extract with distilled water. The total phenolic content, expressed as mg of gallic acid equivalents (GAE) per g of dry beet root pomace extract, was determined using the calibration curve of gallic acid standard.

Total flavonoids. Total flavonoids were determined using the colorimetric assay developed by Zhishen *et al*. (1999). An aliquot (1 ml) of extract (concentration 1 mg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled H_2O. Into the flask, 0.3 ml 5% $NaNO_2$ was added and 5 min later 0.3 ml 10% $AlCl_3$ was added. After 6 min, 2 ml of 1M NaOH solution was added and the total volume was made up to 10 ml with distilled H_2O. The solution was well mixed and the absorbance was measured at 510 nm against the control that had been prepared in the same manner only with replacing the extract with distilled water. Total flavonoid content was expressed as mg rutin equivalents (RE) per g of dry extract.

Total betalain content. The total betalain (betacyanin and betaxanthin) pigment content in the extract was measured spectrophotometrically. The wavelengths of 535 nm and 476 nm were used for betacyanin and betaxanthin analysis, respectively (von Elbe 2003). The aqueous extracts were diluted with 0.05M phosphate buffer (pH 6.5) to obtain the absorption values A_{538} of $0.4 < A < 0.5$. The absorbance was read at 476, 538, and 600 nm. The calculations were carried out according to the following equations: $x = 1.095\ (a - c)$; $y = b - z - x/3.1$; and $z = ax$, where: a = absorbance at 538 nm; b = absorbance at 476 nm; c = absorbance at 600 nm; x = absorbance of betacyanin; y = absorbance of betaxanthin; and z = absorbance of impurities. The contents of betacyanin (BC) and betaxanthin (BX) were calculated using the equations:

$$BC\ (g/100\ ml) = 1000 \times x \times F/A_{1\%,betanin}$$

$$BX\ (mg/100\ ml) = 1000 \times y \times F/A_{1\%,vulgaxanthin\text{-}I}$$

where:

F – dilution factor

$A_{1\%,vulgaxanthin\text{-}I}$, $A_{1\%,betanin}$ – extinction coefficients representing the absorption of 1% solution (1 g/100 ml), being 1120 for betanin and 750 for vulgaxanthin-I

Total betacyanin content was expressed as mg of betanin equivalents per g of dry extract, and total betaxanthin content was expressed as mg of vulgaxanthin-I equivalents per g of dry extract.

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity. The free radical scavenging activity of the extracts was determined spectrophotometrically. The hydrogen atom or electron donation abilities of the extract was measured from the bleaching of a purple-coloured methanol solution of stable 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$). Briefly, 1 ml of solution containing from 0.1 to 0.5 mg of extract in 95% methanol or 1 ml of methanol (control) were mixed with 3 ml of 90μM DPPH solution (18 mg in 50 ml 95% methanol prepared daily) and 8 ml of 95% methanol. The mixture was vortexed thoroughly for 1 min and left at room temperature for 60 min, than the absorbance was read against control at 515 nm.

The control probe contained all components except the radicals. The capability to scavenge the DPPH radicals, DPPH$^{\bullet}$ scavenging activity ($SA_{DPPH\bullet}$), was calculated using the following equation:

$$SA_{DPPH\bullet}\ (\%) = (A_{control} - A_{sample})/A_{control} \times 100$$

where:

$A_{control}$ – absorbance of the control reaction (containing all reagents except the extract)

A_{sample} – absorbance in the presence of the extract

Reducing power. The reducing power of the extracts was determined by the method of Oyaizu (1986). The capacity of the extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purpose, the suspensions of extracts (0.05–1 mg) in 1 ml of distilled water or 1 ml of distilled water (control) were mixed with 1 ml of phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide $K_3[Fe(CN)_6]$. The mixture was incubated at 50°C for 20 minutes. Following this, 1 ml of trichloroacetic acid (10%) was added and the mixture was then centrifuged at 3000 rpm for 10 minutes. A 2 ml aliquot of the upper layer was mixed with 2 ml of distilled water and 0.4 ml of 0.1% $FeCl_3$, and the absorbance of the mixture was measured at 700 nm. The absorbance at 700 nm was used as the indicator of the reducing power. Increased absorbance of the reaction mixture indicated increased reduction capability. Ascorbic acid was used as positive control.

Hydroxyl radical scavenging activity. As hydroxyl free radicals ($^{\bullet}OH$) are highly reactive, with relatively short half-lives, the concentrations found in natural systems are usually inadequate for the direct detection by ESR (electron spin resonance) spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem. Hydroxyl radicals are identified by means of their ability to form nitroxide adducts (stable free radicals form) from the commonly used DMPO (5,5-dimethyl-1-pyroline-*N*-oxide) as the spin trap. The Fenton reaction was conducted by mixing 0.2 ml of 0.3M DMPO, 0.2 ml of 10mM H_2O_2, and 0.2 ml of 10mM Fe^{2+} (control). The influence of different ethanol extracts on the formation and stabilisation of hydroxyl radicals was investigated by adding the investigated extracts in the Fenton reaction system in the concentration range 0.01–0.5 mg/ml. ESR spectra were recorded after 5 min, with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 0.512 G, receiver gain 5×10^5, time constant 81.92 ms, conversion time 327.68 ms, center field 3,440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C.

The $SA_{\bullet OH}$ value of the extract was defined as:

$$SA_{\bullet OH} = 100 \times (h_0 - h_x)/h_0\ (\%)$$

where:

h_0, h_x – heights of the 2nd peak in the ESR spectrum of DMPO-OH spin adduct of the control and the probe

Superoxide anion radical scavenging activity. Superoxide anion radicals ($O_2^{\bullet -}$) were generated in the reaction system obtained by mixing 500 µl of dry dimethylsulfoxide (DMSO), 5 µl of KO_2/crown ether (10mM/20 mM) prepared in dry DMSO, and 5 µl of 2M water solution of DMPO as the spin trap. The influence of the ethanol extracts on the formation and transformation of superoxide anion radicals was determined by adding dimethylforamide (DMF) solutions of the beet root extract fractions to the superoxide anion reaction system in the final range of concentrations 0.01–1 mg/ml (probe). After that, the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The ESR spectra were recorded on an EMX spectrometer from Bruker (Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 4.00 G, receiver gain 1×10^4, time constant 327.68 ms, conversion time 40.96 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW and temperature 23°C.

The $SA_{O_2^{\bullet -}}$ value of the extract was defined as:

$$SA_{O_2^{\bullet -}} = 100 \times (h_0 - h_x)/h_0\ (\%)$$

where:

h_0, h_x – heights of the 2nd peak in the ESR spectrum of DMPO-OOH spin adduct of the control and the probe

Antibacterial activity. For the evalution of the antibacterial activity of the samles, agar disc diffusion method was used. The strains were grown on Mueller-Hinton agar slants at 37°C for 24 h and checked for purity. After the incubation, the cells were washed off the surface of agar and suspended in sterile physiological solution. The number of cells in 1 ml of suspension for inoculation measured by McFarland nefelometer was 5×10^7 CFU/ml.

1 ml of this suspensions was homogenised with 9 ml of melted (45°C) Mueller-Hinton agar and poured into Petri dishes. On the surface of the agar, 6 mm diameter paper discs (HiMedia®, Mumbai, India) were applied and impregnated with 15 µl of samples. The final concentrations of the extracts investigated were 10, 20, and 50 mg/ml. The plates were incubated 48 h at 37°C and the diameters of the resulting zones of inhibition (ZI) were measured and expressed in mm. The evaluation of the antimicrobial activities of the samples was carried out in triplicates.

Microbroth dilution method was performed according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS 2000) on 96-well microplates (Spektar, Čačak, Serbia). Methanol solutions of the ethanol extract of beet root pomace (20 µl) were added to the wells with 80 µl Müller-Hinton broth (Torlak, Belgrade, Serbia) containing 5×10^6 CFU/ml of each bacteria. The covered plates were incubated under aerobic conditions at 37°C for 24 hours.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no visible growth could be detected.

In parallel with the antibacterial investigation pure solvent was also tested, exhibiting no antibacterial activity (data are not shown).

Statistical analysis. All measurements were carried out in triplicates, and were presented as means ± SD. Regression analysis and significance of differences were determined using Origin 7.0 Software package (OriginLab Corporation, Northampton, USA, 1991–2002). Statistical significance level was fixed at $P < 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

The evaluation of the composition of beet root pomace extracts was based on five representative indices: total phenolics (TPh), total flavonoids (TFl), total betaxanthins (TBx), and total betacyanins (TBc) contents (Table 1). The ethanolic extract showed the highest phenolic (376.4 mg/g expressed as mg of gallic acid equivalents per g of dry beet root pomace extract) and betalaine (41.85 mg/g, total betacyanin content was expressed as mg betanin equivalents per g of dry extract and total betaxanthin content was expressed as mg vulgaxanthin-I equivalents per g of dry extract) contents while the highest flavonoid (269.70 mg/g expressed as mg rutin equivalents per g of dry extract) content was found in the acetone extract. The amounts of total phenolics and flavonoids were the lowest in the water extract while the amount of total betalains in the acetone extract.

DPPH radical scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods (Duh *et al*. 1999; Chang *et al*. 2002). The effect of antioxidants on DPPH radical scavenging was thought to result from their hydrogen donating ability. DPPH$^\bullet$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in methanol was at 517 nm. It is visually noticeable as a discoloration from purple to yellow. Figure 1 shows the dose response curve for the radical scavenging effect of the ethanol (E1), acetone (E2), and water (E3) extracts. The scavenging of DPPH radicals increased with increasing extract concentration.

Table 1. Composition of beet root pomace extracts after SPE

Extracts	TPh (mg/g)	TFl (mg/g)	TBx (mg/g)	TBc (mg/g)
Ethanol	376.4** ± 18.82	253.50 ± 12.55**	17.67 ± 0.82	24.18 ± 1.19*
Acetone	343.8 ± 17.15**	269.70 ± 13.21**	11.19 ± 0.51	22.65 ± 1.12*,**
Water	218.3 ± 10.72	200.50 ± 10.00	22.90 ± 1.11	18.78 ± 0.91**

TPh – total phenolics expressed as mg of gallic acid equivalents per g of dry beet root pomace extract; TFl – total flavonoids expressed as mg rutin equivalents per g of dry extract; TBx – total betaxanthins was expressed as mg vulgaxanthin-I equivalents per g of dry extract; TBc – total betacyanins expressed as mg betanin equivalents per g of dry extract
Data are the mean ± SD of three experiments (significantly different at the level $^{*}P < 0.05$ or $^{**}P < 0.01$, ANOVA)

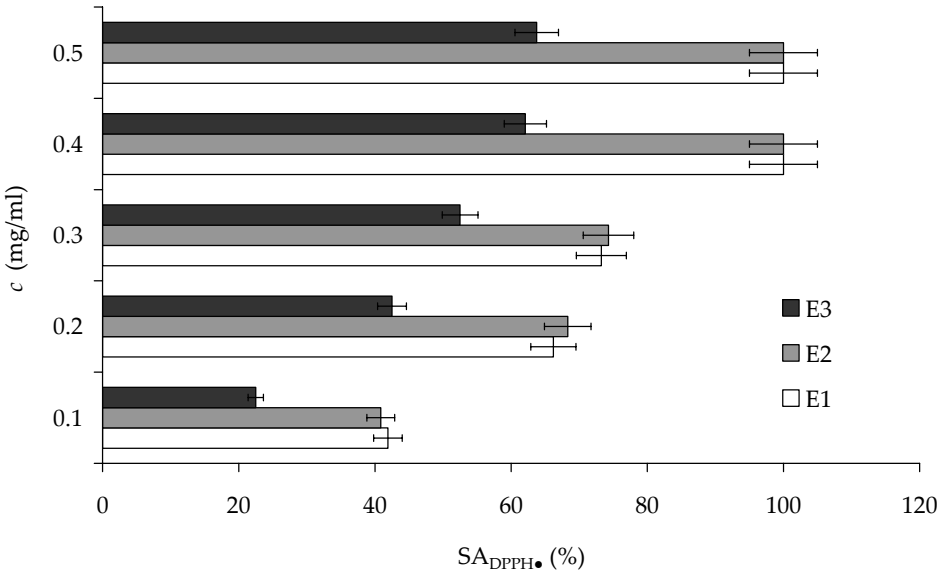

Figure 1. The scavenging activity ($SA_{DPPH•}$) of different concentrations of ethanol (E1), aceton (E2) and water (E3) beet root pomace extracts on DPPH radicals

The EC_{50} value, defined as the concentration of the extract required for 50% scavenging of radicals under the experimental condition employed, is a parameter widely used to measure the free radical scavenging activity (Cuvelier *et al*. 1992); a smaller EC_{50} value corresponds to a higher antioxidant activity.

The EC_{50}^{DPPH} values of beet root pomace extracts, determined on the basis of radical scavenging activities ($SA_{DPPH•}$) of the extracts, are presented in Table 2. It was observed that ethanol and aceton extracts posses the same high level of DPPH radical scavenging activity, while the water extract was less effective in DPPH test. When BHA was tested as the pure reference compound, the EC_{50}^{DPPH} = 0.028 ± 0.001 mg/ml.

Reducing power

For the measurements of the reducing power, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of the beet root pomace extracts using the method of Oyaizu (1986). The reducing power is associated with the antioxidant activity and may serve as a significant indicator of the antioxidant activity (Oktay *et al*. 2003). However, the antioxidant activities of antioxidants have been attributed to various mechanisms, among which are the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive kapacity, and radical scavenging (Diplock 1997; Yildirim *et al*. 2000). Figure 2 shows that the reducing powers of the ethanol (E1), acetone (E2), and water (E3) extracts increased with increasing concentrations of all extracts. Also in this test, the ethanol and aceton extracts of beet root pomace exerted the same level of reducing power.

Table 2. EC_{50} values of the extracts against DPPH radicals[a]

Beet root pomace extract	EC_{50}^{DPPH} (mg/ml)
Ethanol	0.133 ± 0.006
Acetone	0.133 ± 0.006
Water	0.275 ± 0.013

[a]Data expressed as mean ± SD

ESR spectroscopic determination of hydroxyl and superoxide anion free radicals scavenging activity of ethanol beet root extracts

Based on the fact that the ethanol extract of beet root pomace possesses very high cotents of all investigated phenols and betalains, the ESR determination of scavenging activity on $^{\bullet}OH$ and $O_2^{\bullet-}$ were applied on that extract. The antioxidant capacities may be determined by measuring and comparing the decrease of ESR peak height in and without the presence of antioxidants using the Fenton Fe^{2+}/H_2O_2 radical or superoxide anion radicals generating system.

Using a spin trap, such as DMPO, it is possible to convert reactive hydroxyl radicals to stable nitroxide radicals (DMPO-OH adducts) with spectral

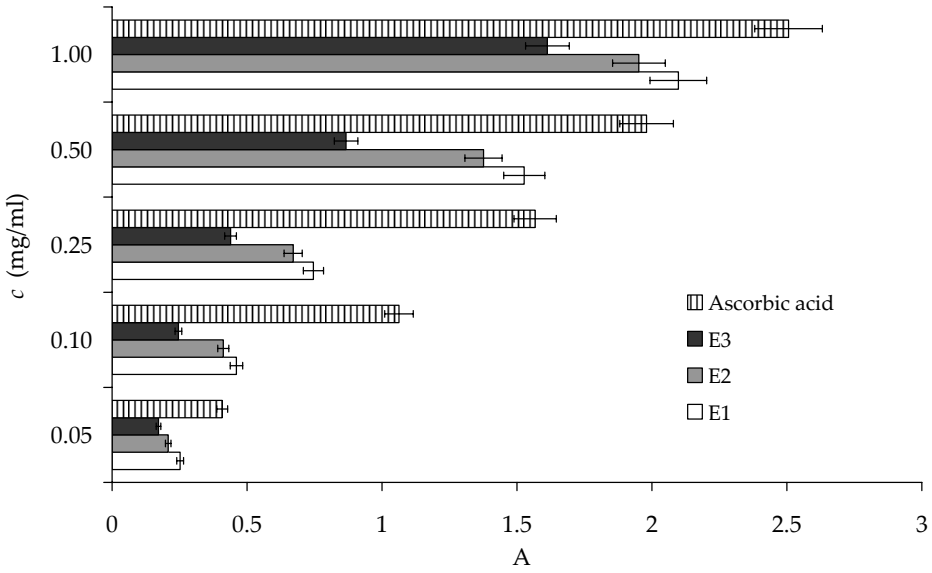

Figure 2. Comparison of the reducing power of different concentrations of beet root pomace extracts by spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformation at 700 nm

hyperfine splitting that reflects the nature and structure of these radicals. The reaction of Fe^{2+} with H_2O_2 in the presence of the spin trapping agent DMPO generated 1:2:2:1 quartet of lines with hyperfine coupling parameters ($a_N = a_H = 14.9$ G). It was shown that the HO production was reliable and stable using the classic Fenton Fe^{2+}/H_2O_2 radical generating system, and the amounts of DMPO/HO formed in this system were proportional to ESR peak height or signal intensity, which was measured on the second peak of the ESR curve in the spectra. The scavenging activity towards hydroxyl radicals increased in the presence of different amounts of beet root extracts (Figure 3).

In the presence of the 0.1 mg/ml of ethanol extract, scavenging activity towards hydroxyl radical was about 50% ($SA_{\bullet OH}$ = 52.53%). Hence, the highest investigated concentration (0.5 mg/ml) of ethanol extract inhibited completely the formation of hydroxyl radical. The value obtained for BHA was EC_{50}^{OH} = 1.505 ± 0.062 mg/ml.

Although superoxide anion is a weak oxidant, it gives rise to the generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Liu *et al.* 2008). Superoxide anion radical can be produced enzymatically, in xanthine/xanthine oxidase system or chemically, as in this study. The advantage of the latter system for the determination of superoxide anion radical scavenging activity is the simplicity in the interpretation of the results, since the activity towards the enzyme is not included. Superoxide anion radical scavenging activities of beet root pomace extracts are presented in Figure 4.

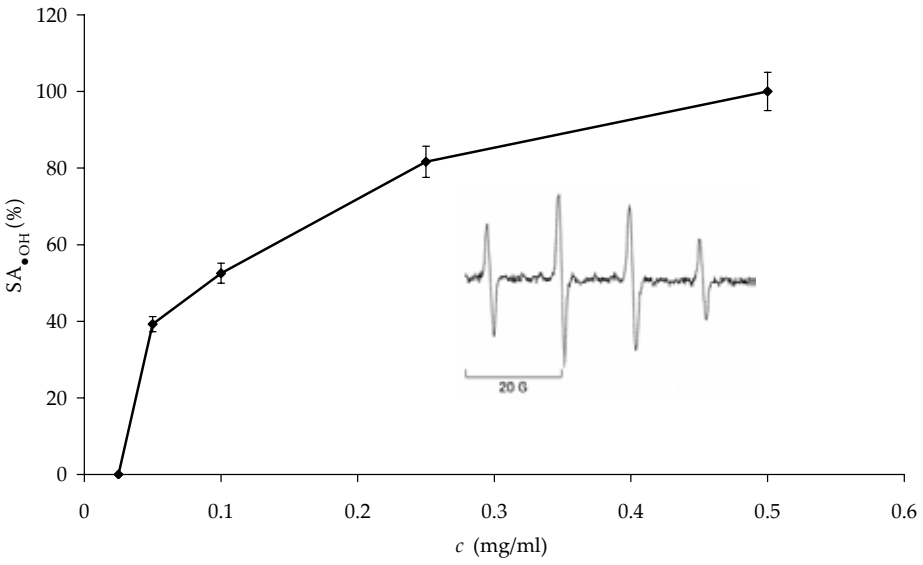

Figure 3. Scavenging effect ($SA_{\bullet OH}$) of different concentrations of ethanol (E1) beet root pomace extract on the DMPO-OH spin adduct during the Fenton reaction

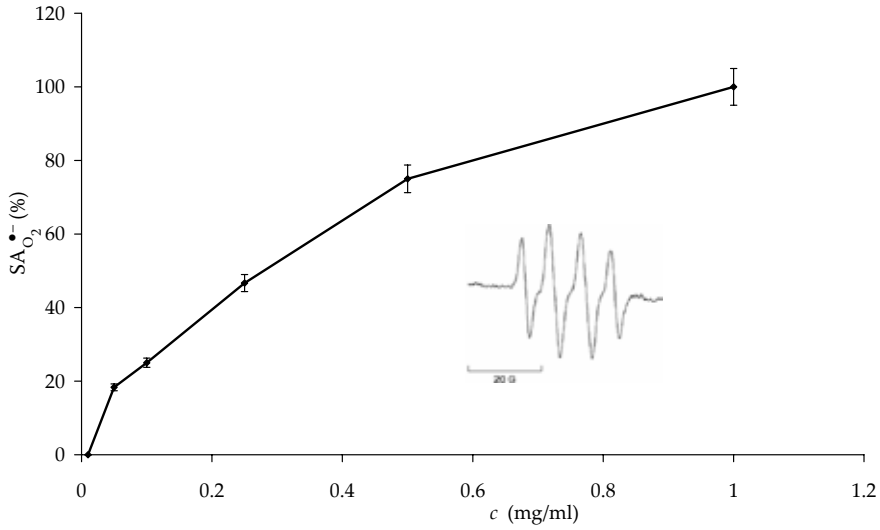

Figure 4. Scavenging effect ($SA_{O_2^{\bullet-}}$) of different concentrations of ethanol (E1) beet root pomace extract on the superoxide anion radical

Ethanol extracts showed a dose response curve for the superoxide anion radical scavenging effect. The maximum activity, 100%, was shown by the extract of the concentration of 1.0 mg/ml. The value obtained for BHA was $EC_{50}^{O_2^{\bullet-}}$ = 2.680 ± 0.125 mg/ml.

It was observed that the investigated ethanol extract of beet root pomace was less effective in superoxide anion scavenging than in •OH radical assay. EC_{50} for hydroxyl radical was 0.082 mg/ml while the EC_{50} value for superoxid anion radical was 0.259 mg/ml, indicating that the ethanol extract of beet root pomace possessed strong antioxidant activities which were higher than the BHA activity. The capacity of beet root pomace to inhibit hydroxyl radical generated by the Fenton reaction could be due to the direct scavenging effect and/or to the inhibition of hydroxyl generation. The second mechanism occurs by ion chelation (Calliste *et al.* 2005). Superoxide reactions with phytochemicals appear to be inversely correlated with their one-electron redox potentials (Larson 1997).

Correlation between composition data and radical scavenging activity

In order to determine the relative importance of various classes of phenolic compounds in free radicals scavenging activity, correlation analyses were made between the values of $SA_{DPPH\bullet}$, $SA_{\bullet OH}$ or $SA_{O_2^{\bullet-}}$ and the phytochemicals contents (Table 3).

A highly significant correlation was observed between the contents of phenolics, flavonoids, and betaxanthins and the DPPH scavenging activity ($r > 0.8$) (Table 3). But the content of betacyanins slightly correlated ($r = 0.33$) with this activity. The antiradical activity of phenolic compounds depends on their molecular structure, that is on the availability of phenolic hydrogens and on the possibility of stabilisation of the resulting phenoxyl radicals formed by hydrogen donation (Rice-Evans *et al.* 1996; Ramarathnam *et al.* 1997).

A high linear correlation was observed between the contents of all investigated phytochemicals and the scavenging of hydroxyl ($r > 0.8$) and superoxide anion radicals ($r > 0.9$) in the extract. There are several mechanisms of the antioxidant activity, but it is believed that radical scavenging via hydrogen atom donation is the predominant mode. Other antioxidant mechanisms established involve radical complexing of pro-oxidant metals as well as quenching through electron donation and singlet oxygen quenching (Calliste *et al.* 2005).

Linear correlation between radical scavenging activity and polyphenolic concentration has been reported in an extensive range of vegetables,

Table 3. Correlation matrix between the results of the phytochemical contents and radicals scavenging activity

Antioxidant compounds	$r_{DPPH\bullet}$	$r_{O_2^{\bullet-}}$	$r_{\bullet OH}$
Total phenolics	0.94	0.92	0.81
Total flavonoids	0.90	0.92	0.80
Total betacyanins	0.33	0.92	0.80
Total betaxanthins	0.88	0.95	0.81

fruits and beverages (Marja *et al*. 1999; Gil *et al*. 2000).

Antibacterial activity

The *in vitro* antibacterial activity of ethanol extract of beet root pomace was evaluated by two methods: disc diffusion method and microdilution method using selected Gram-positive and Gram-negative bacteria. The diameter of the inhibition zone (ZI) and minimum inhibitory concentration (MIC) are shown in Table 4.

The data indicate that the extract exhibited the activity against the investigated food pathogens. Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* demonstrated higher susceptibility than Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. An important fact is that the extract showed antibacterial activity against *Staphylococcus aureus* (MIC = 0.75 mg/ml), one of the most common gram-positive bacterium causing food poisoning. On the other hand, a weak antimicrobial activity was found against *Escherichia coli* (MIC = 1.5 mg/ml) and *Pseudomonas aeruginosa* (MIC = 4.5 mg/ml). It is also important to note that this typical soil and water bacterium *Pseudomonas aeruginosa,* showed the lowest sensibility to the antibiotic Chloramphenicol.

Phenolic compounds possess high levels of antimicrobial activity (Baydar *et al*. 2004), e.g. carvacrol, oxygenated derivatives (thymol methyl ether) and its precursors *p*-cymene and *γ*-terpinene (Skočibušić *et al*. 2006). Most of the studies on the mechanism of phenolic compounds focused on their effects on cellular membranes, altering their function and in some instances their structure, causing swelling and increasing their permeability. The increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, decreased ATP levels, and the loss of the proton motive force, which lead to cell death.

Table 4. Diameter of the inhibition zone (ZI) and minimum inhibitory concentrations (MIC) of ethanol extract of beet root pomace[a]

Bacteria	Ethanol extract of beet root pomace		Chloramphenicol
	ZI (mm)	MIC (mg/ml)	MIC (μg/ml)
Escherichia coli	8 ± 0.0	1.5	5.0
Pseudomonas aeruginosa	n.d.	4.5	50.0
Staphylococcus aureus	8 ± 1.0	0.75	5.0
Bacillus cereus	10.3 ± 0.58	0.5	10.0

[a]Data are means ± SD (n = 3); n.d. not detected

CONCLUSION

This study indicates that the extracts obtained from beet root pomace possess considerable amounts of phenolic compounds and betalains and a significant radical scavenging activity towards stable DPPH and highly reactive hydroxyl and superoxide anion radicals. The correlation coefficients exhibited a positive relationship between the antiradical activities of beet root pomace and the contents of total phenolics, flavanoid, anthocyanin, and betaxanthins.

Our results showed that beet root pomace, an inedible waste product in juice manufacture, might be a potent source of antioxidants, and has a potential as a value-added ingredient for functional foods.

More work should be done to characterise the individual phytochemical compounds in the extracts of beet root pomace in order to assign particular antioxidant effects to individual compounds of the extracts. In future studies, it would be desirable to employ such experimental conditions that can more specifically reflect the *in vivo* antioxidant activities of the extracts obtained.

References

Azeredo H.M. (2009): Betalains: properties, sources, applications, and stability – a review. International Journal Food Science and Technology, **44**: 2365–2376.

Baydar H., Sagdic O., Ozkan G., Karadogan T. (2004): Antibacterial activity and composition of essential oils from *Origanum*, *Thymbra* and *Satureja* species with commercial importance in Turkey. Food Control, **15**: 169–172.

Calliste C.A., Trouillas P., Allais D.P., Duroux J.L. (2005): Castanea sativa mill. Leaves as new sources of natural antioxidant: An electron spin resonance study. Journal of Agricultural and Food Chemistry, **53**: 282–288.

Chang L.W., Yen W.J., Huang S.C., Duh P.D. (2002): Antioxidant activity of sesame coat. Food Chemistry, **78**: 347–354.

Cuvelier M.E., Richard H., Berset C. (1992): Comparison of the antioxidative activity of some acid-phenols: Structure–activity relationships. Bioscience, Biotechnology and Biochemistry, **56**: 324–325.

Diplock A.T. (1997): Will the good fairies please prove us that vitamin E lessens human degenerative disease? Free Radical Research, **27**: 511–532.

Duh P.D., Tu Y.Y., Yen G.C. (1999): Antioxidant activity of water extract of Harng Jyur (*Chrysanthemum morifolium* Ramat). Food Science and Technology, **32**: 269–277.

Francis F.J. (1999): Colorants. Eagan Press, St. Paul: 55–66.

Gil M.I., Tomas-Barberan F.A., Hess Pierce B., Holcroft D.M., Kader A.A. (2000): Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. Journal of Agricultural and Food Chemistry, **48**: 4581–4589.

Kähkönen M.P., Hopia, A.I., Vuorela H.J., Rauha J.P., Pihlaja K., Kujala T.S., Heinonen M. (1999): Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry, **47**: 3954–3962.

Kaur C., Kapoor H.C. (2001): Antioxidants in fruits and vegetables – the millennium's health. International Journal Food Science and Technology, **36**: 703–725.

Kujala T., Loponen J., Pihlaja K. (2001): Betalains and phenolics in red beetroot (Beta vulgaris) peel extracts: extraction and characterisation. Zeitschrift für Naturforschung – C, **56**: 343–348.

Kujala T.S., Loponen J.M., Kika K.D., Pihlaja K. (2000): Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. Journal of Agricultural and Food Chemistry, **48**: 5338–5342.

Larson A. (1997): Naturally Occurring Antioxidants. Lewis Publishers, New York.

Liu X., Zhao M., Wang J., Yang B., Jiang Y. (2008): Antioxidant activity of methanolic extract of emblica fruit (*Phyllanthus emblica* L.) from six regions in China. Journal of Food Composition and Analysis, **21**: 219–228.

Makris D.P., Boskou G., Andrikopoulos N.K. (2007): Polyphenolic content and *in vitro* antioxidant characteristics of wine industry and other agri-food solid waste extracts. Journal of Food Composition and Analysis, **20**: 125–132.

Marja P.K., Anu I.H., Heikki J.V., Jussi-Pekka R., Kalevi P., Tytti S.K., Marina H. (1999): Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry, **47**: 3954–3962.

NCCLS (2000): Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 5th Ed. NCCLS Document M7-A5. NCCLS, Wayne.

Ness A.R., Powles J.W. (1997): Fruit and vegetables, and cardiovascular disease. International Journal of Epidemiology, **26**: 1–13.

Oktay M., Gülçin İ., Küfrevioğlu Ő.İ. (2003): Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Food Science and Technology, **36**: 263–271.

Oyaizu M. (1986): Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition, **44**: 307–315.

Pedreno M.A., Escribano J. (2001): Correlation between antiradical activity and stability of betanine from *Beta vulgaris* L roots under different pH, temperature and light conditions. Journal of the Science of Food and Agriculture, **81**: 627–631.

Ramarathnam N., Ochi H., Takeuchi M. (1997): Natural antioxidants; chemistry, health effects and applications. AOCS Press, Champaign: 76–87.

Riboli E., Norat T. (2003): Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. American Journal of Clinical Nutrition, **78**: 559–569.

Rice-Evans C.A., Nicholas J.M., George P. (1996): Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology & Medicine, **20**: 933–956.

Rigo A., Vianello F., Clementi G. (2000): Contricution of proanthocyanidins to the peroxy radical scavenging capacity of some Italia red wines. Journal of Agricultural and Food Chemistry, **48**: 1996–2002.

Skočibušić M., Bezić N., Dunkić V. (2006): Phytochemical composition and antimicrobial activities of the essential oils from *Satureja subspicata* Vis. growing in Croatia. Food Chemistry, **96**: 20–28.

Schieber A., Stintzing F.C., Carle R. (2001): By-products of plant food processing as a source of functional compoundssrecent developments. Trends in Food Science & Technology, **12**: 401–413.

Schilderman P.A.E.L., ten Vaaewerk F.J., Lutgerink J.T., Van der Wurf A., ten Hoor F., Kleinjans J. (1995): Induction of oxidative DNA damage and early lesions in rat gastro-intestinal epithelium in relation to prostaglandin H synthase-mediated metabolism of butylated hydroxyanisole. Food and Chemical Toxicology, **33**: 99–109.

Singleton V.L., Orthofer R., Lamuela-Raventós R.M. (1999): Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, **299**: 152–178.

Vinson J.A., Hao Y., Su X., Zubik L. (1998): Phenol antioxidant quantity and quality in foods: vegetables. Journal of Agricultural and Food Chemistry, **46**: 3630–3634.

von Elbe J.H. (2003): Current Protocols in Food Analytical Chemistry. John Wiley & Sons, New York: F4.1.1–F4.1.9.

Wang M., Goldman I.L. (1997): Transgressive segregation and reciprocal effect for free folic acid content in a red beet (*Beta vulgaris* L.) population. Euphytica, **96**: 317–321.

Will F., Bauckhage K., Dietrich H. (2000): Apple pomace liquefaction with pectinases and cellulases: Analytical data of the corresponding juices. European Food Research and Technology, **211**: 291–297.

Yildirim A., Mavi A., Oktay M., Kara A.A., Algur O.F., Bilaloglu V. (2000): Comparison of antioxidant and antimicrobial activities of tilia (*Tilia argenta* Desf Ex DC), sage (*Salvia triloba* L.) and black tea (*Camellia sinensis*) extracts. Journal of Agricultural and Food Chemistry, **48**: 5030–5034.

Zhishen J., Mengcheng T., Jianming W. (1999): The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry, **64**: 555–559.

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