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ANTIOXIDANT PROPERTIES OF ETHANOLIC EXTRACT OF SUGAR BEET PULP

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The ethanolic extract of sugar beet pulp at 100% exibited good antioxidant activity of 69.74%. It showed an excellent scavenging activity on 1,1-diphenyl-2-picrylhydrazyl radicals (84.94%) and was a mild chelator for ferrous and cupric ions. On the basis of some identified phenolic acids in ethanolic extract from sugar beet pulp it is concluded that predominant acids (ferulic, gentisic and p-coumaric acid), which have been previously evidented as relatively potent antioxidants, contribute to the antioxidant properties of the investigated extract. Antioxidative nature of ethanolic extract from sugar beet pulp, that is sugar beet pulp itself, indicate that it could be used as antioxidative component for decreasing oxidative changes in foods.

KEYWORDS: Sugar beet pulp; ethanolic extract; antioxidant activity; scavenging effect; chelating effect

INTRODUCTION

Sugar beet pulp is a by-product which is obtained in technological procedure of sugar beet processing in sugar refining industry. Sugar beet pulp is primarily used as a component in final feedstuffs. However, its chemico-nutritional composition, that is the importance of high content of its dietary fiber, sugests the possibility of use of sugar beet pulp as a raw material for high ability hydrating aditive production (1) or production of dietary fiber, vanillin and some other products which can be applied in many branches of the food industry (2, 3).

Although the classifing of sugar beet pulp in a group of raw materials for functional food production primary results from its high dietary fiber content, the information about content of ferulic acid in sugar beet pulp (4), as well as about its antioxidant effect (5-9) and its antifungal nature (10) point out the possibility of complex effects of sugar beet pulp or products obtained from this material in funtional food.

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The statements about antioxidant nature of ferulic acid are of great importance if we have in mind that oxygen free radicals that can be generated both in biosystems and food react with the wide range of biomolecules (lipids, proteins, DNA) provoke their changes and damages (11-13). The protection of an organism from oxygen free radicals implies activity of enzymatic (catalase, SOD, glutathione peroxidase, glutathione reductace, etc.) and nonenzymatic (vitamin E, vitamin C, glutathione, uric acid, etc.) systems of antioxidative protection. Disturbance of the balance between production of oxygen free radicals (or some other radical species) and activity of antioxidative system of protection causes the so-called oxidative stress (14), which further results in numerous deseases – atherosclerosis (15, 16), rheumatoid arthritis (17), Crohn's disease and ulcerative colitis (18), cancer (11, 19), nephrological and immuno diseases (20) and others.

The tolerance of milder form of oxidative stress in biosystems or decrease or elimination of oxidative changes of lipids in food processing among other things implies the application of antioxidants, while application of natural antioxidants has being favoured during the last decades because of the evidences about toxicity of synthetic antioxidants (21). The abundance of statements referring to the evidence that one of the most numerous classes of antioxidants are polyphenols, including phenolic acids, as well as having in mind the presence of antioxidative potent ferulic acid in sugar beet pulp (4), determine that the aim of this work was the examination of antioxidant properties of ethanolic extract from sugar beet pulp. The estimation of antioxidant nature of this extract has been investigated by monitoring its antioxidant activity, scavenging activity on DPPH radicals and chelating effects on metal ions ($Fe^{2+}$and Cu^{2+}). In adition, the identification and quantification of phenolic acids from ethanolic extract, which have been reported to contribute to the antioxidant properties of the examinated extract, have been carried out.

EXPERIMENTAL

Ethanol, methanol and chloroform were products of "Zorka", Šabac, Serbia and Montenegro. Methanol (HPLC grade), acetonitrile (HPLC grade), gallic acid, potassium chloride, ferrous sulphate and copper sulphate were products of Merck & Co., Inc., Germany. The 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Denis reagent, β-carotene, linoleic acid, Tween 40, α-tocopherol, butylated hydroxytoluole (BHT), tetramethyl murexide and standards of phenolic acids (caffeic acid, chlorogenic acid, *trans*-cinnamic acid, *p*-coumaric acid, ferulic acid, gentisic acid and protocatechuic acid) were products of Sigma Chemical CO, St. Louis, MI. Hexamine was product of Aldrich Chemical Company, Inc. These chemicals were of analytical reagent grade.

Sugar beet pulp. Commercially obtained sugar beet pulp was ground in a Condux-Werk mill type LS10K. The mass was sieved through the set of sieves and the fraction which passed throught a 200 μ screen was taken for further investigations. The composition of the sugar beet pulp was analyzed using the methods of AOAC (method number 7.007 for moisture – 6.89%; 7.015 for crude protein – 8.96%; 7.061-7.065 for crude fiber – 18.00%; 7.056-7.057 for crude fat – 1.21%; 7.009 for mineral matters – 4.28%; 7.097-7.098 for calcium – 0.76%; 7.120-7.123 for phosphorus – 0.08%; hemicellulose – 18.30%; 7.072 for lignin – 15.72%; 7.073 for reduced – 0.70% and total sugars – 6.83%, respectively) (22).

Extractions. Sugar beet pulp (4 g) was mixed with 40 mL of 80% ethanol. Extraction was carried out with shaking at room temperature during 1 h. Extract was separeted by filtering through the filter paper (Whatman, Grade 4 Chr, UK), and procedure was repeated with 40 mL of ethanol two times. Ethanolic extracts (3 × 40 mL) were combined, and solvent was removed under vacuum at 40°C to obtained 25 mL volume. The extract obtained by following this procedure was used for further investigtions of antioxidant activity.

Extraction of phenolic acids for qualitative and quantitative analysis was carried out according to the procedure descreibed in the work of Emmons and Peterson (23). Four subsamples (1.0 g) of sugar beet pulp were extracted with 80% ethanol (3 × 10 mL, 20 min, with shaking) at room temperature. Following centrifugation (5 min at 1,250 × g), the supernatants were filtered through filter paper (Whatman, Grade 4 Chr, UK), combined, and solvent was removed under vaccum at 40°C. Phenolic compounds were resolubilized in 5 mL methanol and filtered throught a 0.45-μm membrane filter (Millipore) prior to chromatography.

Determination of total phenolic compounds. The total phenolic compounds present in sugar beet pulp were determined spectrophotometrically using Folin-Denis reagent (22). The ethanolic extract (0.1 mL) of sugar beet pulp in volumetric flask was diluted with distilled water (75 mL). Folin-Denis reagent (5 mL) was added, and the contents of the flask were mixed thoroughly. After 3 min, Na_2CO_3 solution (10 mL; concentration 10 g/100 mL) was added and finally quantified to 100 mL with distilled water. The mixture was allowed to stand for 30 min with intermittent shaking. The blue colour was measured with a spectrophotometer (6405 UV/VIS, Jenway). The concentration of total phenolic compounds in sugar beet pulp was determined by comparison with the absorbance of standard gallic acid at different concentrations.

Antioxidant activity assay. Antioxidant activity was measured by monitoring the coupled autoxidation of β-carotene and linoleic acid (23, 24). β-carotene (2 mg) was dissolved in 20 mL of chloroform, and 3 mL of this solution were added to 40 mg of linoleic acid and 400 mg of Tween 40. The chloroform was removed under steam of N_2 gas. Oxygenated deionized water (100 mL) was added and the solution was mixed well. Aliquots (3 mL) of β-carotene and linoleic acid emulsion were mixed with 40 μL of ethanolic extracts (diluted to various concentrations – 100, 75, 50, 25, 12.5 and 6.25%) and incubated in a water bath at 50°C. Oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm at 15-min intervals for 60 min. Controls contained 40 μL of ethanol instead of the extracts. Antioxidant activity (AOA) was expressed as percent of inhibition relative to the control after 60-min incubation period (25).

$$AOA = 100(DR_C - DR_S) / DR_C$$

where: DR_C = degradation rate of control = ln(a/b)/60; DR_S = degradation rate of sample = ln(a/b)/60; a and b = absorbance at 0 and 60 min.

Activities of extracts were compared with the activities of a natural antioxidant, α-tocopherol, and a synthetic antioxidant, butylated hydroxytoluole (BHT), which were used at 20 mM as controls.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals. Various concentrations of sugar beet pulp (100, 75, 50, 25, 12.5 and 6.25%) (4 mL) were mixed with 1 mL of

methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, such that the final concentration of DPPH was 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min, then the absorbance was measured at 517 nm (26).

Chelating effects on ferrous and cupric ions. The chelating effect of ethanolic extract of sugar beet pulp was measured according to the method of Shimada and co-workers (26). To 2 mL of the mixture, consisting of 30 mM hexamine, 30 mM potassium chloride and 9 mM ferrous sulphate (or copper sulphate), various concentration of etnanolic extract (100, 75, 50, 25, 12.5 and 6.25%) (2 mL) and tetramethyl murexide (0.2 mL, 1mM) were added. The absorbance of the mixture was measured at 485 nm after 3 min at room temperature. A lower absporbance of the reaction mixture indicated a higher chelating ability.

Reversed-phase HPLC. Phenolic compounds were separated by reversed-phase HPLC. Samples (10 μL) were injected onto a C_8 reversed-phase column (Hypersil MOS, 5μm, 200 × 2,1 mm, Hewlett Packard), eluted for 60 min with a linear gradient of 1-40% acetonitrile in water, adjusted to pH 2.8 with acetic acid (flow rate 0.200 mL/min), and monitored at 290 nm. Peaks were identified by comparing retention times and UV scans (200-400 nm), using a diode array detector (model 79880 DAD), with known standards (caffeic acid, chlorogenic acid, *trans*-cinnamic acid, *p*-coumaric acid, ferulic acid, genistic acid, protocatechuic acid). Peak identities were further verified by adding internal standards to the sample. Quantitation was achieved by comparing peak areas with external standards.

RESULTS AND DISCUSSION

The ethanolic extract of sugar beet pulp (100%) exibited good antioxidant activity, i.e. lower than that of 20 mM α-tocopherol and 20 mM BHT, while antioxidant activity of series of different concentrations of ethanolic extract from sugar beet pulp (75-12.5%) decreased with the increased dilution (Table 1). If relative high content of total phenols of sugar beet pulp is put into consideration (0.45% as gallic acid), that is of extract from sugar beet pulp (100%) (0.16 mg/mL as gallic acid), antioxidant activity of various concentrations of the investigated extract could be attributed to phenols which:

- decrease concentration of peroxy radicals by donating H-atom that result in formation of less reactive *cis,trans*-hydroperoxides

$$(LOO^{\bullet} + ArOH \rightarrow LOOH + ArO^{\bullet});$$

- decrease concentration of peroxy radicals by interaction with aroxy radicals – scavenging effect

$$(LOO^{\bullet} + ArO^{\bullet} \rightarrow \text{products})$$

- and inhibit formation of peroxy radicals by the mechanism of complexing of metal ions.

With the aim to explain the demonstration of antioxidant activity of the investigated series of different concentrations of ethanolic extract from sugar beet pulp identification and quantification of some phenolic acids which were present in the extract were carried out by HPLC technique (Fig. 1, Table 2).

Table 1. Antioxidant activity (AOA) of ethanolic extract from sugar beet pulp

Sample	Concentration	Antioxidant activity (AOA)
α-tocopherol	20 mM	87.62
BHT	20 mM	85.82
Ethanolic extract of sugar beet pulp	100%	69.74
	75%	64.68
	50%	61.90
	25%	41.36
	12.5%	32.37
	6.25%	13.02

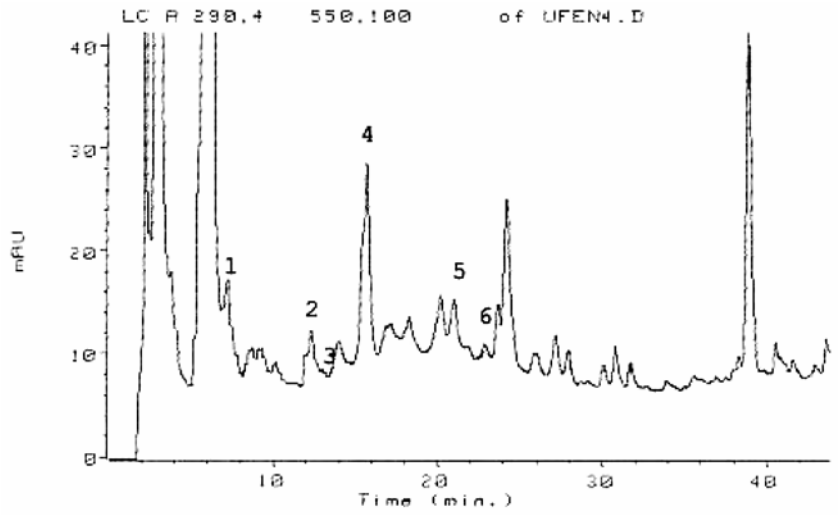

Fig. 1. Separation of phenolic components from a representative sample of ethanolic extract of sugar beet pulp by reversed-phase HPLC on C_8 reversed-phase column using a 1-40% acetonitrile gradient, pH 2.8, detected at 290 nm. Peaks are: (1) gentisic acid, (2) chlorogenic acid, (3) caffeic acid, (4) *p*-coumaric acid, (5) ferulic acid, and (6) cinnamic acid

Table 2. Contents of some phenolic acids identified in ethanolic extract of sugar beet pulp

Compound	Content (mg/kg)
Protocatechuic acid	0.000
Gentisic acid	2.954
Chlorogenic acid	1.375
Caffeic acid	0.063
Ferulic acid	5.983
Cinnamic acid	1.487
p-Coumaric acid	3.039

The content of ferulic acid which were found in ethanolic extract from sugar beet pulp (5.983 mg/kg) is significantly lower than its content in sugar beet pulp (≈ 8 mg/g) (4), that could be explained if facts about association of ferulic acids with the side chains of pectins are taken into consideration, so it is neccesary to apply hydrolysis, for example enzymatic, to release them in free and esterified forms (27).

All identified phenolic acids in ethanolic extract from sugar beet pulp belong to the group of antioxidant compounds (28, 29). The predominant acids from extract (ferulic, gentisic and *p*-coumaric acid) (Table 2) have been known as relatively potent antioxidants, that possess the structural elements that could be responsible for their antioxidant potency (6, 9, 30). Considering the structure of gentisic acid, which possesses two OH-groups (*ortho-metha*), it is the most potent antoxidant from all identified phenolic acids in ethanolic extract from sugar beet pulp (6). Chlorogenic acid, which also was found in ethanolic extract from sugar beet pulp was identified earlier as an antioxidant which could act as DPPH radical scavenger and inhibitor of formation of conjugated dienes (30).

The series of different concentrations of ethanolic extract of sugar beet pulp showed excellent scavenging activity on DPPH radicals at concentrations from 50-100% (Table 3), which was caused by donating H-atoms from available hydroxyl groups of phenolic acids in ethanolic extract (26).

Scavenging effect of 100% ethanolic extract of sugar beet pulp (84.94%) in comparison with the effect of BHT (93.50%) and α-tocopherol (83.01%) (Table 3) showed their similar activities as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Table 3. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical

Sample	Concentration	Scavenging effect (%)
α-tocopherol	20 mM	83.01
BHT	20 mM	93.50
Ethanolic extract of sugar beet pulp	100%	84.94
	75%	81.05
	50%	75.92
	25%	48.27
	12.5%	28.83
	6.25%	12.11

Based on the results of investigation of a series of antioxidants Brand-Williams and co-workers (7) revealed that caffeic, gentisic and ferulic acids exibited powerful antiradical activity on DPPH radicals, which also was confirmed by Son and Lewis (31). Ohnishi and co-workers (30) found that chlorogenic acid was DPPH radical scavenger too, while Kikuzaki and co-workers (9) cited next hierarchy order conserning the phenolic acids as DPPH scavengers: caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid. Based on the measurement of disappearance of methyl linoleate in dodecane during heating at 110°C (6), as well as on monitoring the formation of hydroperoxides on 100°C the same order of antioxidative activities was registered.

The levels of scavenging effects on DPPH radicals which were achieved by the application of the series of different concentrations of ethanolic extract of sugar beet pulp on the system for measuring antiradical activity were in accordance with those achieved on the system for measuring the antioxidant activity. This indicated that the antioxidant

activity of investigated extract could be caused in the great part by donating H-atoms from phenolic acids in extract to peroxy radicals, which is favoured by the resonance stabilization of phenoxy radicals. Since stabilization of phenoxy radicals depends on the presence and number of OH-groups in aromatic ring, as well as on the presence of electron-donating CH_3O-groups in *ortho* position (29) demonstrated antiradical activity on DPPH radicals caused by phenolic acids in the examined extract was completely clear, even more if the dominant acids in the extract were taken into consideration (Table 2).

The components of ethanolic extract of sugar beet pulp also acted as metal chelators, that is they supressed cathalytic lipid peroxidation by complexing metal ions (Fe^{2+} and Cu^2), although not so strongly as α-tocopherol (Table 4). The possibility of complexing metal ions, as the antioxidant properties themselves, depends on structure of compound, that is in the case of phenolic acid implies the number and position of OH- and CH_3O-groups. It can be concluded on the basis of the analysis of structures of phenolic acids which were identified in ethanolic extract of sugar beet pulp that potential metal chelators could be caffeic, ferulic and chlorogenic acids. Ferulic acid, as the predominant acid in the investigated extract, is probably a weak metal chelator, considered *ortho* position of its OH- i OCH_3-group, that could explain relatively weak effect of ethanolic extract of sugar beet pulp on the supresion of catalytic lipid peroxidation. Since Fe^{2+} and Cu^{2+} are the most effective pro-oxidants in food systems (32) the determined effects of chelating on Fe^{2+} and Cu^{2+} (Table 4), no matter they were not pronounced, contributed to total antioxidant potency of ethanolic extract of sugar beet pulp.

Table 4. Chelating effect on Fe^{2+} and Cu^{2+}

Sample	**Concentration**	**Chelating on Fe^2 (%)**	**Chelating on Cu^{2+} (%)**
α-tocopherol	20 mM	94.36	96.18
BHT	20 mM	37.68	29.54
Ethanolic extract of sugar beet pulp	100%	35.42	38.15
	75%	32.29	31.75
	50%	17.51	13.16
	25%	0	0
	12.5%	0	0

The obtained results show that ethanolic extract of sugar beet pulp, that is sugar beet pulp itself, possesses strong antioxidant activity, which could be borne in mind during the tolerance of mild oxidative stress in biosystems or during decreasing or eliminating of oxidative changes in food lipids and food processing if sugar beet pulp would be used, for example, as raw material for biomass production.

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АНТИОКСИДАТИВНЕ ОСОБИНЕ ЕТАНОЛНОГ ЕКСТРАКТА РЕПИНОГ РЕЗАНЦА

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Етанолни екстракт излуженог самлевеног репиног резанца (100%) остварио је антиоксидативну активност од 69,74%. Наведени екстракт испољио је и одличну антирадикалску активност на 1,1,-дифенил-2-пикрилхидразил радикале (84,94%), док је његов хелатизирајући ефекат на Fe^{2+} и Cu^{2+} био релативно слаб.

На основу присуства неких фенолних киселина идентификованих у испитиваном етанолном екстракту закључено је да су доминантне киселине (ферулна, гентистична и *п*-кумаринска), од раније познате као релативно потентни антиоксиданти, одговорне за антиоксидативна својства испитиваног екстракта. Антиоксидативна природа етанолног екстракта репиног резанца, односно и самог репиног резанца, указује на могућност његовог коришћења као антиоксидативне компоненте за смањивање оксидативних промена у храни.

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