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Antioxidant Activity and Polyphenol Content of *Cranberries (Vaccinium macrocarpon)*

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Abstract: Cranberries (*Vaccinium macrocarpon*) contain many bioactive compounds and have some biological activities and beneficial health properties. In the study, antioxidant effects of lyophilized aqueous extract of cranberry (LAEC) and quantity of some its polyphenolic compounds were determined. For this purpose, we performed $DPPH^{\cdot}$, $DMPD^{\bullet+}$, $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ radicals scavenging activities, inhibition of lipid peroxidation activity by thiocyanate method, Cu^{2+} and Fe^{3+} reducing abilities, FRAP assay and Fe^{2+} binding activity. At the 10 µg/mL concentration, LAEC inhibited 52.4% lipid peroxidation produced by linoleic acid emulsion. Also, α-tocopherol, BHA, trolox, and BHT had 52.5, 89.9, 93.1 and 94.9% inhibition value at 30 µg/mL concentration, respectively. Quantitative amounts of some phenolic compounds in LAEC were investigated by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). *p*-Hydroxy benzoic acid was found as the most abundant phenolic compound (55 mg/kg extract) in LAEC.

Keywords: Cranberries (*Vaccinium macrocarpon*); Polyphenol content; Radical scavenging; Antioxidant activity; LC-MS/MS.

1. Introduction

Cranberries (*Vaccinium macrocarpon*) grow in North America and are processed for food products such as juice, sweetened dried cranberries, sauce, and some pharmaceutical products [1]. They possess a lot of phenolic compounds [2,3]. It is well known that these phenolics are responsible for a variety of health benefits, including anticancer activity [4,5], antioxidant capacity can prevent platelet aggregation and LDL oxidation [6]. Berries are notable sources of natural phenols in human diet and associated with the health-promoting effects of vegetables and fruits. They can act as protective agents against chronic diseases such as cardiovascular and heart disease, cancer and many degenerative diseases. The bioactive components in cranberry have been well characterized [7,8]. These phytochemicals act as antioxidants and neutralizing of free radicals damage. Cranberries had anti-adhesion properties and were used for prevention of urinary tract infections, gum disease and stomach ulcers [9,10].

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Free radicals and reactive oxygen species (ROS), which oxidize important cellular components constantly, occurred in biological systems [11]. On the other hand antioxidants can eliminate ROS and prevent important cellular components from oxidation [12]. Also, ROS play crucial preventive roles in the development of many chronic diseases including cardiovascular diseases, aging, heart disease, diabetes, inflammation, anaemia, degenerative diseases, cancer, ischemia and cancer [13,14]. Antioxidants are molecules, which had defensive effects against ROS in the body [15]. In addition, there is a growing trend in consumer preferences towards natural antioxidants [16].

In this study, we investigated the inhibition of lipid peroxidation, Fe^{3+}, Cu^{2+} and Fe^{3+}-TPTZ reducing powers, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$and $O_2^{\bullet-}$ scavenging and Fe^{2+} chelating activities of LAEC.

2. Materials and Methods

2.1. Chemicals

Ferrozine (3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine), Neocuproine (2,9-dimethyl-1,10-phenanthroline), ABTS (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), DMPD (N,N-dimethyl-p-phenylenediamine), BHA (butylated hydroxyanisole), $DPPH^{\cdot}$ (1,1-diphenyl-2-picryl-hydrazyl) and were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). The compounds were used as standards in LC-MS/MS analysis obtained from Fluka or Sigma-Aldrich.

2.2. Preparation of Lyophilized Aqueous Extract of Cranberry (Vaccinium macrocarpon)

Extraction procedure was described in detail previously [17,18]. For LAEC, 25 g of cranberry (*Vaccinium macrocarpon*) was ground into a fine powder using a blender. The cranberry powder was mixed with 400 mL of boiling water by magnetic stirrer for 15 min. The extract was filtered over cheesecloth and Whatman paper, respectively. The filtrates were collected and frozen at -84°C. Finally, the filtrates are lyophilized in a lyophilizator under 5 mm-Hg pressure at -50°C (Labconco, Freezone, Japan).

2.3. Determination of total phenolic content in LAEC

The quantity of total phenolics was determined by Folin-Ciocalteu method [19] with slight modification [20,21]. The total phenolic content in LAEC was determined based on a standard gallic acid curve and expressed as gallic acid equivalent (GAE) micrograms.

The quantity of total flavonoid contents of LAEC was estimated using the aluminium chloride colorimetric method [22,23]. The total flavonoids content in LAEC was estimated from the standard quercetin curve and expressed as quercetin equivalents (QE) micrograms.

2.4. Preparation of test solution for LC-MS/MS

A portion of LAEC (100 mg) was dissolved in 5 mL of deionised ethanol-water (50:50 v/v). Then 1 mL of this solution was transferred into a volumetric flask, which contains 100 µL of 13C p-hydroxy benzoic acid. The last solution was diluted with ethanol-water (50:50 v/v). From this solution an aliquot (1.5 mL) was transferred into a capped autosampler vial and 10 µL of sample was injected to LC and kept at 15^{o}C during the experiment [24].

Standard mixture of *p*-hydroxy benzoic, vanillin, p-coumaric acid, ascorbic acid, caffeic acid, ferulic acid, ellagic acid, syringic acid, quercetin, apigenin, kaempferol, epigallocatechin, kaempferol-3-glucoside and luteolin-7-glucoside in the range of 100 - 5000 µg/L in methanol were used for calibration purpose [24].

Quantitative amounts of *p*-hydroxy benzoic acid, vanillin, p-coumaric acid, ascorbic acid, caffeic acid, ferulic acid, ellagic acid, syringic acid, quercetin, apigenin, kaempferol, epigallocatechin, keampferol-3-glucoside and luteolin-7-glucoside were detected in LAEC by using liquid

chromatography and tandem mass spectrometry (LC-MS/MS). *p*-Hydroxy benzoic acid was used as an internal standard in the assay [24].

2.5. Liquid Chromatography-Mass spectrometry

The analysis was performed on Zivak Tandem Gold Triple quadrupole mass spectrometry equipped with Macherey-Nagel Nucleoder C18 Gravity (150 x 2 mm i.d. 5 µm particle size). The mobile phase used was A: methanol, 0.1% formic acid B: Water + 0.1% formic acid. The applied gradient program was as follow: 3 mins 100 % B, from 3.01 to 9.0 mins 30 % A and 70 % B, and from 13.01 to 20.0 100 % B.

The applied method validated considering to repeatability, linearity, precision, recovery and LOD/LOQ parameters. The sources and quantification of the uncertainty for the applied method were evaluated. Main inputs of uncertainty budget are determined as impurity of reference standards, sample weighing, calibration curve and dilution of solutions. Detailed validation and uncertainty procedures and explanations were given in the literatures [24].

2.6. Antioxidant Assays

The ferric thiocyanate method was the first method for evaluation of antioxidant effect of LAEC. This method, namely the prevention of peroxidation of linoleic acid emulsion was described previously [25]. In this method, peroxides are formed during the linoleic acid peroxidation. Peroxides lead to oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}). The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm [26]. Low absorbance value at wavelength indicated high level of inhibition of lipid peroxidation and therefore high antioxidant activity. The inhibition percentage of LAEC on lipid peroxidation in linoleic acid emulsion was estimated by following formula: A(%) = 100-(As/Ac x100) where A is inhibition of lipid peroxidation, λc is the absorbance of the control reaction, λs is the absorbance of sample of LAEC [27].

Fe^{3+}-Fe^{2+} reducing power of LAEC and standards was determined according to the method described by Ak and Gülçin [27]. Ferric ions (Fe^{3+})-ferrous ions (Fe^{2+}) reduction is determined by measuring the absorbance of Perl's Prussian blue complex. The cupric ion (Cu^{2+}) reducing power of LAEC was determined by the method proposed by Apak et al. [28] with slight modification [29]. Another used reducing power assay is FRAP, which is based upon reduction of Fe^{3+}-TPTZ complex under acidic condition and described previously [30].

Ferrous ions (Fe^{2+}) chelating activity of LAEC was measured by inhibiting the formation of Fe^{2+}-ferrozine complex after treatment of LAEC with Fe^{2+}, following the method of Dinis et al. [31] described previously [32]. Metal chelating ability of LAEC was monitored by the absorbance of the Fe^{2+}-ferrozine complex at 562 nm [33].

The DPPH free radical scavenging activity of LAEC and standards was evaluated by the method of Blois [34]. The DMPD radical scavenging ability of LAEC and standards was determined by the method of Gülçin [35,36]. The superoxide radical scavenging activity of LAEC and standards was determined by the riboflavin-methionine-illuminate assay. This assay is based on the capacity of the extract to inhibit the photochemical reduction of NBT [37,38].

The percentage of metal chelating and radical scavenging abilities of LAEC and standards were determined using the following equation: Metal chelating or radical scavenging (%) = (1-As/Ac)x100 where λ_c is the absorbance of control and λ_s is the absorbance in the presence of LAEC or standard compounds [39,40].

3. Results and Discussion

Phenolic compounds, ubiquitous in plants are an essential and crucial part of the human diet. They are of considerable interest due to their biological activity including antioxidant properties [24]. The content of phenolic compounds in LAEC was found to be 26.0 µg GAE/g(LAEC). On the other hand, 7.06 µg QE/g(LAEC) was measured in the same LAEC sample.

According to LC-MS/MS experiment, p-coumaric acid, *p*-hydroxy benzoic acid, kaempherol-3-O-glucoside, caffeic acid and ellagic acid were found to be the main phenolic compounds lyophilized aqueous extract of cranberry, respectively (Table 1). On the other hand, vanillin, gallic acid, ascorbic acid, ferulic acid, syringic acid, apigenin and luteoline-3-O-glucoside content have not been determined in LAEC. Validation and uncertainty parameters for phenolic compounds were given in a previous study [24].

Table 1. Quantity of antioxidants and LC-MS/MS parameters of selected compounds in LAEC at the mg/kg concentration.

No	Compounds	Parent Ion	Daughter Ion	Collision Energy (V)	Amount of antioxidants in the extracts (mg/kg)**
1	p-Hydroxy benzoic acid	136.8	92.9	10	55.0
2	Vanillin	151.0	135.5	10	<LOD
3	p-Coumaric acid	163.0	118.7	10	13.0
4	Ascorbic acid	175.0	114.6	12	<LOD
5	Caffeic acid	179.0	134.0	11	5.0
6	Ferulic acid	193.0	177.5	10	1.8
7	Ellagic acid	301.0	150.5	10	3.0
8	Syringic acid	197.0	181.6	10	<LOD
9	Quercetin	301.0	178.6	10	<LOD
10	Epigenin	268.6	116.6	25	<LOD
11	Kaempherol	285.0	143.0	33	1.1
12	Epigallocatechin	305.0	124.0	25	<LOD
13	Kaempherol-3-*O*-glucoside	447.0	284.0	20	11.0
IS*	^{13}C *p*-Hydroxy benzoic acid	137.9	92.9	10	

*: ^{13}C *p*-Hydroxy benzoic acid was used as internal standard,
**: The uncertainty of results should be calculated according to Reference 24.

Ferric thiocyanate assay monitored the amount of lipid peroxide formed during incubation. As can seen in Table 2, LAEC exhibited effective antioxidant activity in the linoleic acid emulsion system. The effect of 10 μg/mL LAEC on lipid peroxidation of a linoleic acid emulsion was found to be 52.5%. On the other hand, BHA, BHT, α-tocopherol and trolox exhibited 89.9, 94.9, 52.5 and 93.1% in the same system, respectively.

Furthermore, LAEC had effective reducing power determined by using the $K_3[Fe(CN)_6]$ and cupric ions (Cu^{2+}) reducing methods when compared to the standards. Also, for measurement of reductive potential of LAEC, Fe^{3+}-Fe^{2+} transformation method [26] was investigated in the presence of LAEC. As can be seen in Table 2, LAEC demonstrated powerful Fe^{3+} reducing ability (r^2: 0.959) with statistically significant differences ($p<0.01$). The reducing power of LAEC and standards increased steadily depending on sample concentration. The reducing power of LAEC and the standard compounds were as follows: BHA (0.666, r^2:0. 919) > BHT (0.775, r^2: 0.919) > α-tocopherol (0.989, r^2: 0.896) > trolox (0.935, r^2: 0.971) ≈ LAEC (0.959, r^2: 0.918). The results demonstrated that reducing power LAEC was higher than standards.

CUPRAC method developed by Apak et al. [28] was frequently used for determination of antioxidant capacity [37]. A cupric ion (Cu^{2+}) reducing ability of LAEC is shown in Table 2, and a positive correlation was observed between the Cu^{2+} reducing ability and LAEC concentrations (r^2: 0.983). Cu^{2+} reducing power of LAEC and standard compounds at the same concentration (30 μg/mL) were as follows: BHT (0.760, r^2: 0.957) > BHA (0.670, r^2: 0.959) ≥ α-tocopherol (0.650, r^2: 0.996) > trolox (0.480, r^2: 0.994) ≈ LAEC (0.430, r^2: 0.983).

As can be seen in Table 2, all standard compounds and LAEC showed marked $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$-$[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ reducing abilities. However, the most powerful $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ reducing power was observed in BHA (2.355, r^2: 0.982). This activity was greater than trolox (2.094, r^2: 0.995) > BHT (1.212, r^2: 0.958) > α-tocopherol (0.428, r^2: 0.908) ≈ LAEC (0.440, r^2: 0.929).

Table 2. Total antioxidant activity, Fe^{3+}, Cu^{2+} and Fe^{3+}-TPTZ reducing abilities, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ scavenging activities and Fe^{2+} chelating activity of LAEC and standards.

Antioxidant assay		BHA	BHT	α-Tocopherol	Trolox	LAEC
Total antioxidant activity	$\lambda_{500}{}^{\Psi}$	89.9	94.9	93.1	52.5	52.4
Fe^{3+}-Fe^{2+} reducing*	λ_{700}	0.666	0.755	0.989	0.935	0.959
	r^2	0.919	0.919	0.896	0.971	0.918
Cu^{2+}-Cu^{+} reducing	λ_{450}	0.670	0.760	0.650	0.480	0.430
	r^2	0.959	0.957	0.996	0.994	0.983
FRAP assay	λ_{593}	2.355	1.212	0.428	2.094	0.440
	r^2	0.982	0.958	0.908	0.995	0.929
DPPH• scavenging **	IC_{50}	6.54	49.50	6.13	57.75	86.63
	r^2	0.813	0.929	0.969	0.883	0.985
$ABTS^{\bullet+}$ scavenging *	IC_{50}	11.75	11.55	46. 20	86.63	99.00
	r^2	0.999	0.997	0.982	0.875	0.943
$DMPD^{\bullet+}$ scavenging **	IC_{50}	26.65	-	-	28.88	24.75
	r^2	0.981	-	-	0.982	0.938
$O_2^{\bullet-}$ scavenging**	IC_{50}	15.40	33.00	36.40	25.67	36.47
	r^2	0.926	0.920	0.905	0.941	0.990
Fe^{2+} chelating**	IC_{50}	43.00	55.13	43.21	31.41	82.69
	r^2	0.973	0.987	0.873	0.962	0.945

*: The values were expressed as absorbance. High absorbance indicates high reducing power ability.

**: IC_{50} values were expressed as μg/mL concentration. Lower IC_{50} values indicate higher radical scavenging activity

$^{\Psi}$: Percentage inhibition effect of LAEC (10 μg/mL) and standards (30 μg/mL) such as BHA, BHT, α-Tocopherol and trolox in linoleic acid emulsion.

LAEC had a strong Fe^{2+}chelating effect. The difference between different concentrations of LAEC (10-20 μg/mL) and the control values were statistically significant ($p<0.01$). IC_{50} value for the Fe^{2+} chelating effect of LAEC was found to be 82.69 μg/mL (r^2: 0.945). Also, the Fe^{2+}chelating effect of LAEC was compared to those of BHA, BHT, α-tocopherol and trolox. On the other hand, IC_{50} values for these standards were found to be 43.0 μg/mL (r^2: 0.973), 55.13 μg/mL (r^2: 0.987), 43.21 μg/mL (r^2: 0.873) and 31.41 μg/mL (r^2: 0.962), respectively. These results show that the Fe^{2+} chelating effect of LAEC was higher than those of BHA, BHT, α-tocopherol and trolox.

Table 2 shows a significant decrease ($p<0.01$) in DPPH radical concentration because of the radical scavenging ability of LAEC and the reference compounds. IC_{50} values for LAEC, BHA, BHT, α-tocopherol and trolox on the DPPH radicals were found as 86.63 μg/mL (r^2: 0.985), 6.54 μg/mL (r^2: 0.813), 49.50 μg/mL (r^2: 0.929), 6.13 μg/mL (r^2: 0.969), and 57.75 μg/mL (r^2: 0.883), respectively. A lower EC_{50} value shows a higher DPPH· scavenging activity [40]. DPPH· scavenging capacity of these samples decreased in the following order: α-tocopherol ≈ BHA > BHT > Trolox > LAEC.

The tested compounds exhibited effective $ABTS^{\bullet+}$ scavenging activity (Table 2). In this study, it was found that LAEC was an effective $ABTS^{\bullet+}$ scavenging effect in a concentration-dependent manner (10-20 μg/mL, r^2: 0.943). EC_{50} value for LAEC in this assay was 99.00 μg/mL. There is a significant decrease ($p < 0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity at all LAEC concentrations (10-20 μg/mL). EC_{50} values for BHA, BHT, α-tocopherol and trolox were found to be 11.75 μg/mL (r^2: 0.999), 11.55 μg/mL (r^2: 0.997), 46.20 μg/mL (r^2: 0.982) and 86.63 μg/mL (r^2: 0.985), respectively. The $ABTS^{\bullet+}$ scavenging effect all samples decreased in the following order: BHT ≈ BHA > α-Tocopherol > Trolox > LAEC.

EC_{50} values belonging to inhibition of superoxide radical ($O_2^{\bullet-}$) scavenging of LAEC and standard antioxidants found as following order: BHA (15.40 μg/mL, r^2: 0.926) < trolox (25.67 μg/mL, r^2: 0.941) < BHT (33.00 μg/mL, r^2: 0.920) < α-tocopherol (36.40 μg/mL, r^2: 0.905) LAEC (36.47 μg/mL, r^2: 0.990). The results showed that EC_{50} values of superoxide radical scavenging of LAEC and α-tocopherol were similar (Table 2).

As shown in Table 2, LAEC was an effective $DMPD^{\bullet+}$ radical scavenger in a concentration-dependent manner (10-30 μg/mL, r^2: 0.938). EC_{50} for LAEC was 24.75 μg/mL. This value was found as 26.65 μg/mL for BHA (r^2: 0.981) and 28.88 μg/mL for trolox (r^2: 0.982). These results clearly showed that $DMPD^{\bullet+}$ radical scavenging activity LAEC was than that of used standard antioxidants

such as BHA and Trolox. There is a significant decrease ($p < 0.05$) in $DMPD^{\bullet+}$ concentration of because of scavenging capacity at all LAEC concentrations. Also, the main drawback of the $DMPD^{\bullet+}$ scavenging method is that its reproducibility and sensitivity dramatically decreased when some hydrophobic antioxidants including α-tocopherol or BHT were used [41].

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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