

BIOACTIVE AND PHARMACOKINETIC CHARACTERISTICS OF PRE-MATURED BLACK RASPBERRY, *RUBUS OCCIDENTALIS*

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ABSTRACT

Black raspberry (BR, *Rubus occidentalis*) is a berry originating from North America. It has a high anthocyanin and flavonoid content that seems to be dependent on ripening. Therefore, the bioactive and pharmacokinetic effects of BR were evaluated using pre-matured BR(PBR) collected from May to June. The total polyphenol, flavonoid and vitamin C content in PBR decreased while anthocyanin increased from 0.13 to 10.70 mg/g at 35 days after fruit set. The antioxidant activities due to DPPH and ABTS determination decreased as BRs were matured. Moreover, it is clear that the content of many phenolic compounds including gallic acid, caffeic acid, p-coumaric acid, ferulic acid, rutin, myricetin, luteolin and kaempferol was diminished during maturing. Both ferulic acid and rutin of BR generated the highest decrease as compared to other phenolic compounds. The pharmacokinetic T_{max} and C_{max} of PBR were 0.6 h and 0.264 $\mu\text{g/mL}$, respectively. Based on these results, PBR can generate qualified functionality as food and/or as a raw medicinal material.

Keywords: black raspberry, anthocyanin, flavonoid, polyphenol, pharmacokinetic

1. INTRODUCTION

Due to concerns for wellbeing and lifestyles of health and sustainability (LOHAS) resulting from a sharply increasing income, the consumption for functional foods is also growing very fast. Many studies have been conducted to develop functional food products containing natural, bioactive compounds to meet consumer demand. Berries – such as blueberry, strawberry, and black raspberry – are used as common functional food additives to meet demand from health-conscious consumers. Black raspberry (BR, *Rubus occidentalis*), which originated from North America, is one of the main functional food additives due to its pharmacological effects that were written about in *Donguibogam*, an ancient Korean medicinal textbook written by J. Hur (CHO *et al.*, 2005). Most of the BR cultivated in Gochang, Korea belongs to *Rubus occidentalis*. However, the BR written of in *Donguibogam*, Korea is *Rubus coreanus* Miquel. BR is normally cultivated in soil with pH 6.0–7.2, and it shows medium water usage (OZGEN *et al.*, 2008). Since BR is generally cultivated in moderate to low incident light on coarsely textured soil with average to low levels of organic matter and inorganic nitrogen, BRs form in June in Korea (BAJCZ, 2014). A morphological study indicates BR is a multi-stemmed shrub that forms broad colonies. BR contains thorns with three (ternate) or five palmately arranged leaflets (LEE *et al.*, 2014). BR seems to generate a bright red flower in May, and it tends to breed a floral leaf that is shorter than its sepal. It has semi-spherical shape with naps. BR berry will turn to wine in color when the berry has matured. Normally, edible BR berries are harvested at the early stage of summer. However, some pre-matured BR berries are also gathered as medicines to improve sexual dysfunction of males, as mentioned in *Donguibogam* (ZHAO *et al.*, 2011).

Approximately 1.7% of anthocyanin in BR tends to be responsible for most BR functions. The main anthocyanins of BR are cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sambubioside, and cyanidin 3-xylosylrutinoside (TIAN *et al.*, 2006; TULIO *et al.*, 2008; LEE *et al.*, 2013). TORRE and BARRITT (1977) have indicated that both cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside of BR anthocyanin seem to contribute to the red or black color and antioxidant effect. In addition, as mentioned above, J. Hur during the Joseon Dynasty indicated that BR (*Rubus coreanus* Miquel) has anti-biotic, anti-cancer, and anaphylaxis effects (BAEK *et al.*, 2005). Such effects have been confirmed by many current scientists (BAEK *et al.*, 2005; LIU *et al.*, 2005; DUNCAN *et al.*, 2009; JEONG *et al.*, 2010; LEE *et al.*, 2011). A study conducted by CHO *et al.* (2005) showed that BR can increase antioxidant activities, and it was confirmed through ABTS, DPPH and TBARS when 1 g BR is added to 100 mL of 10 or 100% ethanol. The study also showed that protocatechuic acid is the most abundant phenolic compound in BR when analyzed *via* HPLC (CHO *et al.*, 2005). CHAE *et al.* (2014) and BOBINAITE *et al.* (2012) demonstrated that BR contains a high amount of ellagic acid. They also reported that ellagic acid of BR possesses strong antioxidant effects (BOBINAITE *et al.*, 2012; CHAE *et al.*, 2014). Moreover, since ellagic acid is not easily metabolized by microflora and/or broken down by stomach acids, it is commonly selected as standard index material for berries.

To increase the use of BR as a functional food additive in Korea, BR was collected and its individual phenolic content was determined in this study. Based on the phenolic content of BR, its antioxidant effects were examined for BR depending on the stage of maturation. In addition, to evaluate the pre-matured BR (PBR, <28 days after fruit set) value and its quality as a functional food additive, the ellagic acid content, which is the standard material index for berries, was evaluated to determine the pharmacokinetic index of PBR.

2. MATERIALS AND METHODS

2.1. Sample preparation

According to research conducted by CHUNG *et al.* (2008), BR planting soil in Gochang, Korea mostly consisted of silt loam (64%), loam (35%) and clay (1%). BRs were cultivated in an open field with soil at pH 5.3~6.7 and harvested from 4-year-old BR plants. All BRs were sampled from May to June of 2014 with an interval of 3-4 days (Fig. 1). The average temperature and humidity of Gochang, Korea during harvest, was 17.4~21.7°C and 72.2~81.0 %, respectively (KMA, 2017). All BRs collected were similar to the Shuttleworth, which was confirmed by the random amplified polymorphic DNA (RAPD).

After harvest of the BR, they were dried at 60°C for 36 h and then powdered for storage. 10 g of BR powder were sampled and mixed with 90 mL double distilled water (DDW). The mixture was distilled twice at 80°C for 2 h. All BR extracts were filtered, freeze dried, and stored at -70°C until use.



Figure 1. Pre-matured black raspberry (PBR, *Rubus occidentalis*) and matured black raspberry fruits harvested from May to June with an interval of 2-3 days.

2.2. Determination of the total phenolics, flavonoids, anthocyanins, and ascorbic acid content

The total phenolics of BR were measured using a modified Folin-ciocalteu colorimetric method (SINGLETON and ROSSI, 1965). 20 μL of filtered BR (1 mg/mL) was mixed with water and Folin-ciocalteu reagent. After 3 min of incubation at room temperature, 300 μL of 20% Na_2CO_3 were added. The mixture was then incubated in the dark for 30 min at

40°C. The absorbance was measured at a wavelength of 725 nm (Beckman Du 730, Beckman Coulter Inc., Fullerton, CA, USA) and expressed as its gallic acid equivalent. The flavonoid content was determined using the method described by MEDA *et al.* (2005) with slight modifications. Briefly, 0.25 mL of BR (1 mg/mL) were mixed with 1 mL DDW, 0.075 mL of 5% NaNO₂, 0.075 mL of 10% AlCl₃, and 0.5 mL of 1 M NaOH. The final volume was then adjusted to 2.5 mL with DDW. The absorbance of each sample was then measured at a wavelength of 410 nm (Beckman Du 730, Beckman Coulter Inc., Fullerton, CA, USA). Quercetin was used as a standard. All readings were expressed as micrograms of quercetin equivalent.

Monomeric anthocyanin was evaluated using the modified pH differential method (CHAOVANALIKIT and WROLSTAD, 2004). Optimum dilution for BR filtrate was defined using 0.025 M of potassium chloride buffer. Individual BR was diluted with either potassium chloride buffer or sodium acetate buffer to reach an optimum state. After 15 min of incubation, the absorbance of each diluted BR was measured at a wavelength of 700 nm. The amount of anthocyanin was calculated as follows:

$$\text{Anthocyanin (mg/g)} = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times 1)$$

A: absorbance of diluted BR = (A_{Avis-max} - A_{700nm}) pH1.0 - (A_{Avis-max} - A_{700nm}) pH 4.5;

MW: expressed as cyanidin 3-glucoside (450);

DF: dilution factor;

ε: molar absorptivity (26,900).

The ascorbic acid content was determined using the modified method described by KAMPFERNKEL *et al.* (1995). Briefly, 0.8 mL of 10 % (w/v) trichloroacetic acid were added to 200 μL of BR filtrate (1 mg/mL). Each mixture was then pre-cooled and centrifuged at 3,000 rpm (4°C) for 5 min. Then, 1.5 mL water and 0.2 mL 0.2 N Folin-ciocalteu were added to 0.5 mL of BR supernatant. After 10 min of incubation at room temperature, the absorbance was recorded at a wavelength of 765 nm. L-ascorbic acid was used as a standard.

2.3. DPPH and ABTS radical scavenging activities

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity of each extract sample was determined using the method described by BRAND-WILLIAMS *et al.* (1995). Briefly, freeze-dried BRs were diluted with methanol to obtain five different concentrations. Each diluted BR (10 μL from 1 mg/mL of BR) was mixed with 0.2 mM of DPPH in dimethyl sulfoxide. After 30 min of incubation at room temperature in the dark, the absorbance was measured at a wavelength of 517 nm using a spectrophotometer. The inhibition percentage was calculated from the equation below:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{Control absorbance} - \text{BR absorbance}) / \text{Control absorbance}] \times 100$$

The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activities of the BR extract were determined using the method described by RE *et al.* (1999). 2.45 mM of potassium persulfate was added to 7 mM of ABTS and the mixture was kept in the dark at room temperature for 12-16 h. The ABTS radical cation solution was diluted with phosphate buffer saline to obtain an absorbance value of less than 0.70 at a wavelength of 734 nm before the analysis. After adding 2.0 mL of diluted ABTS radical cation solution to 20 μL of BR sample (1 mg/mL), the reaction mixture was incubated in a cuvette at 30°C for 6 min. Trolox, an analog of vitamin E antioxidant, was used as control.

The ABTS radical scavenging capacity of the BR was calculated using the following equation:

$$\text{ABTS free radical scavenging activity (\%)} = \frac{\text{[(Control absorbance - BR absorbance)]}}{\text{Control absorbance}} \times 100$$

All DPPH and ABTS values calculated using Graphpad Prism 5.0 (Graphpad Prism Version 5.0, Graphpad Software Inc., San Diego, CA, USA), were generated as the 50% inhibition concentration (IC_{50}).

2.4. Liquid Chromatography analysis for the phenolic quantification and ellagic acid identification

Five g of BR were homogenized in 20 mL of 80 % acetone containing 0.2 % formic acid for 1 min. BR was then concentrated, mixed with 10 mL of acidified water, and passed through an activated C18 Sep-Pak cartridge (Waters Corp., Milford, MA). All adsorbed phenolics onto the C18 column were then recovered with 2 mL of acidified methanol containing 3% formic acid. Individually recovered BR in methanol was filtered, and 10 μ L of BR methanol extract was used to analyze the phenolics using liquid chromatography (LC) (Acquity H-class, Waters) equipped with an autosampler/injector and photodiode Array (PDA) detector. A Shiseido Capcellpak C18 UG (5 μ m, 4.6 \times 250 mm) was used for separation. Both mobile phase A (0.2 M ortho-phosphoric acid, pH1.57) and B (20% 50 mM ammonium dihydrogen phosphate, pH2.6 in 80% acetonitrile) were used for elution. The flow rate was 1.0 mL/min, and detection was performed at 280 nm (gallic acid), 320 nm (caffeic acid, p-coumaric acid, ferulic acid) and 360 nm (rutin, myricetin, luteolin, kaempferol). A gradient was employed as follows: 0-10 min, 80% A; 10-15 min, 70% A; 15-20 min, 60% A; 20-25 min, 10% A; 25-30 min, 10 % A; 30-40 min, 95% A. All phenolics were identified and quantified using external standards (gallic acid, caffeic acid, p-coumaric acid, ferulic acid, rutin, myricetin, luteolin, and kaempferol). Data scanned at 280–360 nm were collected. All LC handling steps were followed as mentioned in a previous study (CHAE *et al.* 2014).

2.5. Animal model and blood sample preparation

Male Sprague Dawley rats (7 wks, 220 g) were obtained from KOSA Bio, Korea. A total of six rats were acclimated in an environmentally controlled breeding room at a temperature of 22 \pm 2°C and relative humidity of 50 \pm 10 % with a 12 h dark/light cycle for one week before being used for the experiments. They were provided *ad libitum* access to commercial chow and water. All rats were handled in accordance with the recommendation of the Regulations for the Administration of Affairs Concerning Experimental Animals (BBRI-IACUC-16001). PBR at a dose of 150 mg/kg was orally administered to each rat. A serial of blood samples was collected at 0, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post oral administration. All blood samples were immediately transferred to a BD vacutainer lithium heparin tube and centrifuged at 3,000 rpm for 10 min at 4°C. The pH of rat plasma (100 μ L) was adjusted to pH 2.5 using 1 M potassium dihydrogen phosphate solution. Serial addition of 50% phosphoric acid and acetonitrile was then performed. The plasma samples were centrifuged at 12,000 rpm for 8 min under refrigerated conditions. The supernatant was taken, dried, and re-dissolved in 0.1 mL methanol. Each supernatant sample was then used for ellagic acid analysis under the LC/MS/MS conditions summarized in Tables 1 and 2.

Table 1. Liquid chromatography (LC)/MS/MS analytical conditions used for analysis of ellagic acid in Sprague Dawley rat serum.

Parameter	Condition		
HPLC system	Agilent 1290 Infinity Binary HPLC system with 6420 triple quadrupole LC/MS system		
Column	Shiseido Capcellpak C18 UG (5 μ m, 4.6 \times 250 mm)		
Column Temperature	40°C		
Flow rate	1.0 mL/min		
Injection volume	10 μ L		
Mobile phase	A : 0.1% Formic acid B : Methanol		
	Time (min) A (%) B (%)		
Gradient step	0	70	30
	7.0	50	50
	13.0	10	90
	15.0	70	30
	18.0	70	30

Ionization Type: ESI (Electrospray ionization) Negative; Gas temperature (°C): 320; Gas flow (L/min): 9; Nebulizer (psi): 15; Capillary (v): 4,000; Scan mode: Multiple reaction monitoring (MRM).

Table 2. Liquid chromatography (LC)/MS analytical conditions used for analysis of ellagic acid in pre-matured black raspberry (PBR, *Rubus occidentalis*) extract.

Compound	RT ¹ (min)	MW ²	Precursor ion (MS m/z)	Product Ion (MS, m/z) Confirm ion	Frag (V)	Collision Energy (V)
Ellagic acid	12.1~12.3	302	30[M-H] ⁻	229 257 185	170	20/16/15

¹RT: Retention time; ²MW: Molecular Weight.

2.6. Pharmacokinetic analysis

5 μ L of different concentrations (0.625, 1.25, 2.5, 5.0, and 10.0 μ g/mL) of ellagic acid in plasma were used to obtain the ellagic acid calibration curve (final concentrations of 31.25, 62.50, 125, 250, 500 ng/mL). As a result of ellagic acid calibration ($R^2 = 0.998$), a linear regression equation was obtained. Each plasma sample was then injected to LC/MS/MS. The pharmacokinetic parameters of ellagic acid were calculated using WinNonlin software version 6.3 on non-compartmental analysis (Pharsight Cor., Mountain View, USA).

2.7. Statistical analysis

All experiments were carried out in triplicates or quadruplicates and expressed as mean \pm standard deviation. Statistical analyses were performed using SPSS program (SPSS version 12.0, SPSS Chicago, IL, USA). Unpaired t-tests or one-way repeated measures ANOVA were performed when appropriate. If significant in the ANOVA test, differences in the

means were determined using Duncan's multiple range tests. Statistical significance was considered when the p -value was less than 0.05.

3. RESULTS

3.1. Quality properties and phenolic compound of *Rubus occidentalis*

The anthocyanin content in BR increased from 0.13 to 10.70 mg/g, although the total polyphenols, total flavonoids, and vitamin C content in BR decreased during maturing (Table 3). The DPPH and ABTS radical scavenging activities of BR collected on June 19th were 8.44 and 4.53 times higher, respectively, compared to those of BR collected on May 23. This indicates that antioxidant activities decreased while maturing. Gallic acid, caffeic acid, p -coumaric acid, ferulic acid, rutin, myricetin, luteolin, and kaempferol were detected in BR (Table 4). Both gallic acid and ferulic acid were found to be its major phenolics. Depending on the BR harvest day, the amount of eight different phenolics decreased ($P < 0.05$) when collected on June 19th, the last day of harvest. Five phenolics (caffeic acid, p -coumaric acid, rutin, myricetin, and kaempferol) had the maximum contents on May 26 or May 30. However, the luteolin content was the highest on June 9th. For this reason, luteolin generated the least disappearance, and only 42.5% of luteolin disappeared when BR was collected on June 19. However, only 1% rutin was left at the end of harvest. The rutin and ferulic acid contents at the end of the harvest were 1 and 3%, respectively. The content decreases for both were the highest compared to the other six phenolics.

3.2. Pharmacokinetic and kinetic analysis

PBR was orally administrated to male Sprague Dawley rats. The plasma concentration-time profile of ellagic acid in Sprague Dawley rats ($n = 5$) is shown in Fig. 2. Ellagic acid was confirmed and quantified when plasma was sampled at 0.25, 0.5, 1, 2, and 4 h after oral administration of PBR.

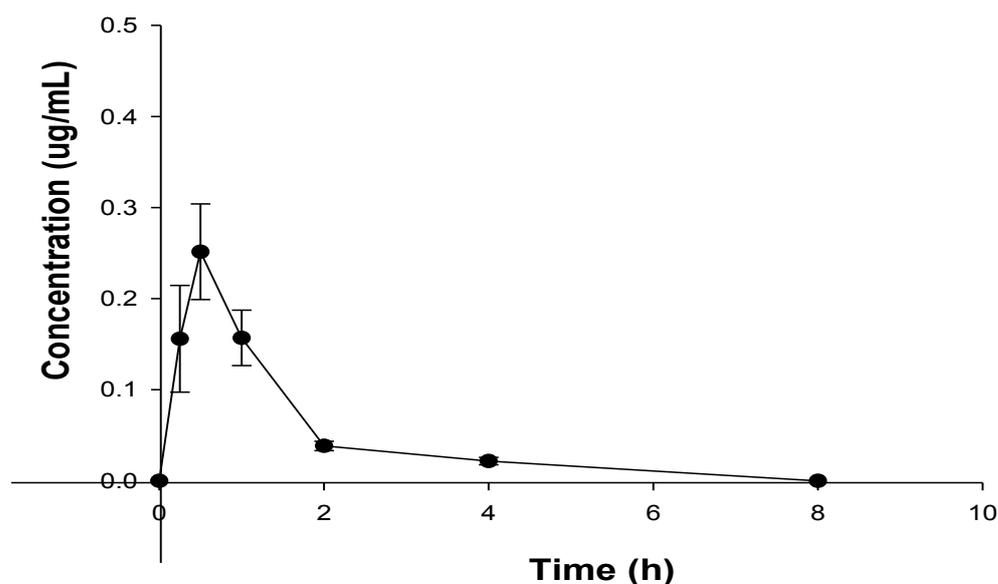


Figure 2. The mean plasma concentration-time profile of ellagic acid in rats after oral administration of pre-matured black raspberry (PBR, *Rubus occidentalis*) at 150 mg/kg (Mean \pm SD, $n = 5$).

Table 3. Chemical quality properties of pre-matured black raspberry (PBR, *Rubus occidentalis*) harvested from late May to early June.

Days After Fruit Set (Date)	Total polyphenol (mg/g)	Total flavonoid (mg/g)	Anthocyanin (mg/g)	Ascorbic acid (mg/100g)	DPPH ² IC ₅₀ (ug/mL)	ABTS ³ IC ₅₀ (ug/mL)
15 (May 23)	170.75±4.65 ^a	1.21±0.02 ^a	0.13±0.01 ^e	24.30±0.38 ^a	95.9±4.52 ^e	261.87±10.25 ^d
18 (May 28)	162.16±4.06 ^a	1.10±0.02 ^b	0.18±0.01 ^e	21.86±0.51 ^b	114.57±4.85 ^{de}	278.13±15.39 ^d
22 (May 30)	148.64±7.78 ^b	1.07±0.04 ^b	0.08±0.01 ^e	20.34±0.14 ^c	126.57±5.69 ^{de}	266.23±9.58 ^d
25 (June 2)	126.52±8.37 ^c	0.80±0.04 ^c	0.12±0.01 ^e	17.03±0.12 ^d	160.67±5.91 ^{de}	326.60±25.54 ^d
28 (June 5)	111.41±2.94 ^d	0.68±0.01 ^d	0.05±0.01 ^e	15.25±0.03 ^e	193.97±1.36 ^d	406.13±31.42 ^d
32 (June 9)	79.04±2.87 ^e	0.51±0.05 ^f	0.81±0.05 ^d	16.79±0.94 ^f	418.83±18.75 ^c	677.73±25.28 ^c
35 (June 13)	60.44±2.62 ^f	0.60±0.01 ^e	3.75±0.12 ^c	10.78±0.03 ^f	824.93±59.99 ^a	912.50±4.90 ^b
38 (June 16)	61.16±7.37 ^f	0.45±0.01 ^f	6.08±0.05 ^b	14.42±0.03 ^g	511.13±57.92 ^b	771.53±99.54 ^{bc}
41 (June 19)	45.15±0.71 ^g	0.52±0.02 ^g	10.70±0.1 ^a	10.62±0.03 ^h	809.77±99.82 ^a	1186.17±294.46 ^a

¹Values are the Mean±SD (n = 3) based on dry basis.

²2,2-diphenyl-1-picrylhydrazyl.

³2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid.

^{a-h}Values with different superscripts within the same columns are significantly different ($p < 0.05$).

Table 4. Phenolic compositions of pre-matured black raspberry (PBR, *Rubus occidentalis*) harvested from late May to early June.

Days After Fruit Set (date)	Phenolic compound (µg/g)							
	Gallic acid	Caffeic acid	<i>p</i> -Coumaric acid	Ferulic acid	Rutin	Myricetin	Luteolin	Kaempferol
15 (May23)	3321.72±1.25 ^a	381.71±52.17 ^{ab}	48.38±46.51 ^{ab}	1733.63±53.32 ^a	326.11±131.49 ^a	63.66±5.18 ^{ab}	2.52±0.13 ^b	11.40±0.31 ^b
18 (May26)	2638.79±6.67 ^b	390.88±72.29 ^a	52.40±22.98 ^a	1260.60±19.55 ^b	398.79±100.58 ^a	60.59±4.34 ^b	2.73±0.05 ^b	13.56±0.38 ^a
22 (May30)	3040.08±0.01 ^c	325.25±65.51 ^{ab}	31.54±13.53 ^{abc}	1241.73±15.96 ^b	324.48±126.41 ^a	70.12±5.70 ^a	2.33±0.23 ^b	11.30±2.26 ^b
25 (June2)	1826.81±0.14 ^d	330.65±53.15 ^{ab}	52.44±5.79 ^a	703.88±6.22 ^c	106.96±77.03 ^b	23.05±5.90 ^d	3.08±0.12 ^b	9.55±0.60 ^{cd}
28 (June5)	1225.55±2.72 ^e	310.03±33.78 ^b	23.17±14.66 ^{abc}	414.35±1.26 ^d	37.89±45.01 ^b	30.16±1.10 ^c	3.44±0.07 ^b	10.18±0.10 ^{bc}
32 (June9)	489.50±1.06 ^f	147.24±9.90 ^c	18.05±0.49 ^{abc}	119.31±5.28 ^e	38.89±14.71 ^b	12.63±2.46 ^e	6.35±3.39 ^a	8.24±0.17 ^d
35 (June13)	373.67±1.23 ^g	21.21±1.50 ^d	15.34±3.63 ^{bc}	77.24±0.32 ^f	46.04±53.14 ^b	13.19±4.02 ^e	3.35±0.15 ^b	4.93±0.43 ^e
38 (June16)	303.98±0.19 ^h	46.84±11.87 ^d	12.64±1.32 ^{bc}	53.15±3.08 ^f	73.47±63.69 ^b	8.79±0.24 ^e	3.22±0.03 ^b	4.47±0.10 ^e
41 (June19)	278.71±3.39 ⁱ	26.74±1.17 ^d	5.98±0.20 ^c	48.30±0.44 ^f	3.68±3.19 ^b	8.66±0.69 ^e	1.45±1.17 ^b	2.08±0.35 ^f

¹Values are the Means ± SD (n = 3) based on dry basis.

^{a-i}Values with different superscripts within the same columns are significantly different ($p < 0.05$).

Ellagic acid was below the detectable level at 8 h after oral administration of PBR. It had the maximum plasma concentration (250 ng/mL) at 0.5 h post oral administration of PBR. The plasma level of ellagic acid decreased as time went by, and its half life ($t_{1/2}$) was 1.018 h. The results of other pharmacokinetic parameters of ellagic acid are summarized in Table 5.

Table 5. Main pharmacokinetic parameters¹ of ellagic acid in rats plasma after oral administration of pre-matured black raspberry (PBR, *Rubus occidentalis*) at 150 mg/kg (Mean \pm SD, n = 5).

	Unit	Mean ²
AUC _{0-t}	ug h mL ⁻¹	0.330 \pm 0.109
AUC _{0-∞}	ug h mL ⁻¹	0.363 \pm 0.118
$t_{1/2}$	h	1.018 \pm 0.195
T _{max}	H	0.600 \pm 0.223
C _{max}	ug mL ⁻¹	0.264 \pm 0.109
MRT	h	1.494 \pm 0.309

¹AUC_{0-t}: Area under the curve, AUC_{0- ∞} : Area under the curve, $t_{1/2}$: half life time, T_{max}: maximum time, C_{max}: maximum concentration, MRT: Mean residence time.

²Each value represents the Mean \pm SD.

4. DISCUSSION

BR was collected during its period of maturation at an interval of 2-3 days. The results for the amount of anthocyanin indicated that anthocyanin may not be a major component in BR contributing to its antioxidant effects before its complete maturation (Table 3). FLOEGEL *et al.* (2011) reported that high-pigmented and hydrophilic BR antioxidants seem to have both DPPH and ABTS scavenging activities, with higher activities toward ABTS than that for DPPH. In a study conducted by LEE *et al.* (2013), seven different anthocyanins were detectable in BR. Only cyanidin 3-glucoside, which has a lower amount compared to cyanidin 3-rutinoside and/or cyanidin 3-xylosylrutinoside, is found to have stronger antioxidant effect (DPPH scavenging activity) than ascorbic acid, but not Trolox (KÄHKÖNEN and HEINONEN, 2003). However, BORGES *et al.* (2010) suggested that the antioxidant activity of raspberries is influenced by ascorbic acid content rather than cyanidin 3-glucoside in this case, they showed 10.5 and 8.1 % antioxidant activity, respectively. In this study, PBR polyphenol and flavonoid contents were found to have significantly diminished with increase in maturation days. The study also indicated that the amount of ascorbic acid also showed a similar trend as evidenced by both polyphenol and flavonoid content. Therefore, in PBR, both DPPH and ABTS seem to be influenced by the amount of polyphenol, flavonoid and ascorbic acid rather than anthocyanin. This is similar to results obtained in a study conducted by OGAWA *et al.* (2008), which suggests that ascorbic acid and flavonols contribute to the antioxidant properties of berries.

In PBR, eight major phenolic compounds were detected. Each phenolic compound showed the least amount when BR was harvested on June 19th (Table 4). A study conducted by KANG *et al.* (2015), showed that whole black raspberry did not contain luteolin and myricetin but catechin, epicatechin, rutin, gallic acid and quercetin were detectable. Although our study did not trace any catechin, epicatechin and quercetin due to different analysis conditions, the amount of rutin was similar to that of fully matured black raspberry. In addition to that, many phenolic compounds of PBR were noted to decrease

during maturing periods. CHIRINOS *et al.* (2007) indicated that the contents of both phenolic compounds and flavan 3-ols during maturity stage of 10 mashua cultivars were different, and diminution of both phenolic compound and flavan 3-ols were attributed due to genotypic difference of mashua. Therefore, among BRs, the Shuttleworth at Gochang, Korea is a cultivar that may decrease some phenolic compounds in maturing stag, therefore, ascorbic acid and flavonols may be major factors that contributed to the DPPH and ABTS scavenging activities of BR at the early stage of the harvest.

To determine the LC protocol accuracy (recovery) and repeatability (precision) for ellagic acid determination in both PBR and rat plasma, ellagic acid was used. The recovery (103.01–104.23 %) and relative standard deviation (RSD, $\leq 3.35\%$) implied that the protocol had satisfactory accuracy and high recovery for ellagic acid. In addition, the RSD of intra- (1.92 %) and inter-day (0.61–2.14 %) differences indicated that the LC protocol was precise. Hence, the LC method seemed to be accurate and precise to quantify ellagic acid in PBR (32.13 ± 0.62 mg/g) (data are not shown). Based on the accuracy and repeatability of LC, ellagic acid plasma concentration-time profile in rats and six major pharmacokinetic parameters were analyzed. The pharmacokinetic results of PBR ellagic acid indicated that PBR ellagic acid in rats seemed to increase rapidly (Table 5), and the C_{max} reached 0.264 $\mu\text{g/mL}$. The half time of ellagic acid was found to be 1.018 h after oral administration. Similar results were reported in a pharmacokinetic study by LEI *et al.* (2003), in which the maximum concentration of pomegranate leaf ellagic acid in rat plasma is found to be 213 ng/mL after 0.55 h of oral administration. The pharmacokinetic characteristics revealed that ellagic acid seems to have poor absorption and rapid elimination after oral administration (SEERAM *et al.*, 2004; BALA *et al.*, 2006). The rapid elimination of ellagic acid has been confirmed *via* urine and feces analysis by SMART *et al.* (1986), with up to 70 % ellagic acid being detected in urine and feces.

5. CONCLUSIONS

This is the first study to characterize pre-matured black raspberry (PBR, *Rubus occidentalis*). PBRs are pre-matured black raspberry when BRs are collected from 15 to 28 days after fruit set. The results showed that PBR under 28 days after the fruit set seemed to produce a high content of phenolic compounds, flavonoids, and vitamin C, thereby possessing excellent antioxidant activities. Based on the PBR pharmacokinetic results and chemical analysis along with antioxidant results, PBR can be used as a functional food or medicinal material derived from the nature. Our data could be used as a baseline to develop nutraceuticals, natural colorants, and other related products.

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REFERENCES

- Baek B.K., Lim C.W., Jung J.M., Choi M.J., Lee S.I., Lee H.K. and Jeon B.H. 2005. Effects of black raspberry wine on testosterone level of New Zealand white rabbits. *Korean J. Orient. Physiol. Pathol.* 19:375.
- Bajcz A.W. 2014. Reproductive ecophysiology of *Rubus occidentalis* in southeastern Michigan I. A survey of tolerance ranges. *Rhodora.* 116:249.

- Bala I., Bhardwaj V., Hariharan S., Kharade S.V., Roy N. and Ravi Kumar M.N. 2006. Sustained release nanoparticulate formulation containing antioxidant-ellagic acid as potential prophylaxis system for oral administration. *J. Drug Target.* 14:27.
- Bobinaite R., Viskelis P. and Venskutonis P.R. 2012. Variation of total phenolics, anthocyanins, ellagic acid and radical scavenging capacity in various raspberry (*Rubus* spp.) cultivars. *Food Chem.* 132:1495.
- Borges G., Degeneve A., Mullen W. and Crozier A. 2010. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants and cranberries. *J. Agric. Food Chem.* 58:3901.
- Brand-Williams W., Cuvelier M.E. and Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci. Technol.* 28:25.
- Chae K.S., Son R.H., Park S.Y., Kim K.A., Lee T.B. and Kwon J.W. 2014. Analytical method validation of ellagic acid as marker compound for standardization of black raspberry extract as functional ingredient. *Food Eng. Prog.* 18:1.
- Chaovanalikit A. and Wrolstad R.E. 2004. Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. *J. Food Sci.* 69:FCT67.
- Chirinos R., Campos D., Arbizu C., Rogez H., Rees J-F., Larondelle Y., Noratto G. and Cisneros-Zevallos, L. 2007. Effect of genotype, maturity stage and post-harvest storage on phenolic compounds, carotenoid content and antioxidant capacity of Andean mashua tubers. *J. Sci. Food Agric.* 87:437.
- Cho Y.J., Chun S.S., Kwon H.J., Kim J.H., Yoon S.J. and Lee K.H. 2005. Comparison of physiological activities between hot-water and ethanol extract of Bokbunja (*Rubus coreanum* F.). *J. Korean Soc. Food Sci. Nutr.* 34:790.
- Chung B-Y., Lee K-S., Kim M-K., Choi Y-H., Kim M-K. and Cho J-Y. 2008. Physico-chemical properties of rainfall interception culture and open field culture soils of *Rubus* sp. in Gochang-gun, Jeollabuk-do. *Korean J. Soil Sci. Fert.* 41:303.
- Duncan F.J., Martin J.R., Wulff B.C., Stoner G.D., Tober K.L., Oberyzyzn T.M., Kusewitt D.F. and Van Buskirk A.M. 2009. Topical treatment with black raspberry extract reduces cutaneous UVB-induced carcinogenesis and inflammation. *Cancer Prev. Res.* 2:665.
- Floegel A., Kim D.O., Chung S.J., Koo S.I. and Chun O.K. 2011. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J. Food Comp. Anal.* 24:1043.
- Jeong J.H., Jung H., Lee S.R., Lee H.J., Hwang K.T. and Kim T.Y. 2010. Anti-oxidant, anti-proliferative and anti-inflammatory activities of the extracts from black raspberry fruits and wine. *Food Chem.* 123:338.
- Kähkönen M.P. and Heinonen M. 2003. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* 51:628.
- Kampfenkel K., Montagu M.V. and Inze D. 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Anal. Biochem.* 225:165.
- KMA-Korea Meteorological Administration. 2017. Weather information. <http://web.kma.go.kr>.
- Kang D-R., Chung Y-H., Shim K-S. and Shin D. 2015. Comparison of chemical properties and phenolic compound for ethanol extract of blueberry, bokbunja and mulberry and their pomace. *Korean J. Organic Agri.* 23:535.
- Lee J., Dossett M. and Finn C.E. 2013. Anthocyanin fingerprinting of true bokbunja (*Rubus coreanus* Miq.) fruit. *J. Funct. Foods.* 5:1985.
- Lee J., Dossett M. and Finn C.E. 2014. Mistaken Identify: Clarification of *Rubus coreanus* Miquel (Bokbunja). *Molecules.* 19:10524.
- Lee K.I., Kim S.M., Kim S.M. and Pyo B.S. 2011. Comparison of fatty acids and antibacterial activity against pathogen of acne in different parts of ripened black raspberry (*Rubus coreanus* Miquel). *J. Korean Soc. Food Sci. Nutr.* 40:466.
- Lei F., Xing D.M., Xiang L., Zhao Y.N., Wang W., Zhang L.J. and Du L.J. 2003. Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract. *J. Chromatogr. B.* 796:189.
- Liu Z., Schwimer J., Liu D., Greenway F.L., Anthony C.T. and Wlotering EA. 2005. Black raspberry extract and fractions contain angiogenesis inhibitors. *J. Agric. Food Chem.* 53:3909.

- Meda A., Lamien C.E., Romito M., Millogo J. and Nacoulma O.G. 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chem.* 91:571.
- Ogawa K., Sakakibara H., Iwata R., Ishii T., Sato T., Goda T., Shimoi K. and Kumazawa S. 2008. Anthocyanin composition and antioxidant activity of the crowberry (*Empetrum nigrum*) and other berries. *J. Agric. Food Chem.* 56:4457.
- Ozgen M., Wyzgoski F.J., Tulio A.Z., Gazula A., Miller A.R., Scheerens J.C., Reese R.N. and Wright S.R. 2008. Antioxidant capacity and phenolic antioxidants of Midwestern black raspberries grown for direct markets are influenced by production site. *HortScience.* 43:2039.
- Re R., Pellegrini N., Proteggente A., Pannala A., Yang M. and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26:1231.
- Seeram N.P., Lee R. and Heber D. 2004. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta.* 348:63.
- Singleton V.L. and Rossi Jr, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144.
- Smart R.C., Huang M-T., Chang R.L., Sayer J.M., Jerina, D.M. and Conney A.H. 1986. Disposition of the naturally occurring antimutagenic plant phenol, ellagic acid, and its synthetic derivatives, 3-O-decylellagic acid and 3,3'-di-O-methylellagic acid in mice. *Carcinogenesis.* 7:1663.
- Tian Q., Giusti M.M., Stoner G.D. and Schwartz S.J. 2006. Characterization of a new anthocyanin in black raspberries (*Rubus occidentalis*) by liquid chromatography electrospray ionization tandem mass spectrometry. *Food Chem.* 94:465.
- Torre L.C. and Barritt B.H. 1977. Quantitative evaluation of Rubus fruit anthocyanin pigments. *J. Food Sci.* 42:488.
- Tulio Jr A.Z., Reese R.N., Wyzgoski F.J., Rinaldi P.L., Fu R., Scheerens J.C. and Miller A.R. 2008. Cyanidin 3-Rutinoside and cyaniding 3-Xylosylrutinoside as primary phenolic antioxidants in black raspberry. *J. Agric. Food Chem.* 56:1880.
- Zhao C., Kim H.K., Kim S.Z., Chae H.J., Cui W.S., Lee S.W., Jeon J.H. and Park J.K. 2011. What is the role of unripe *Rubus coreanus* extract on penile erection? *Phytother. Res.* 25:1046.

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