Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Effect of Plant Part, Extraction Method, and Harvest Time over Antioxidant Yield of *Rubus coreanus*

Min Ju Lee, Ju Hee Nam, Jeong Ho Jeong[1], Il Rae Rho[2]

Division of Applied Life Science (Department of Agronomy), Gyeongsang National University, Jinju, [1]Protected Horticulture Research Institute, National Institute of Horticultural and Herbal Science, Rural Development Administration, Haman, [2]Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, South Korea

Submitted: 13-Dec-2019 Revised: 26-Dec-2019 Accepted: 17-Mar-2020 Published: 30-Nov-2020

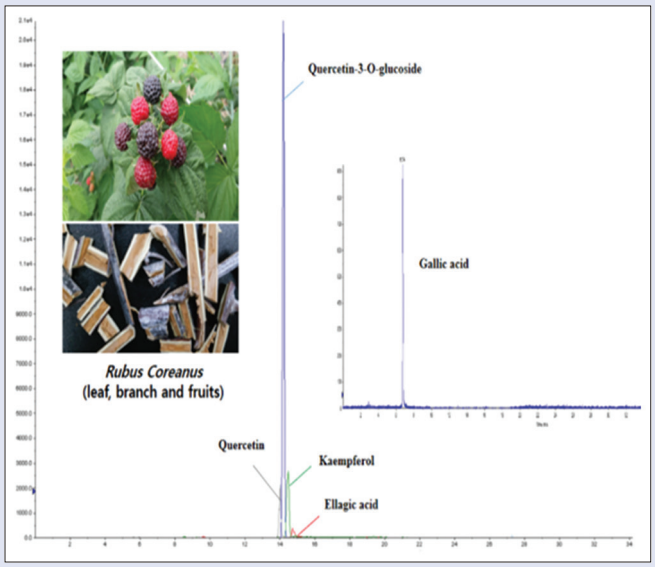
ABSTRACT

Background: Immature fruit of *Rubus coreanus*, also known as "Bokbunja" in Korea, have been used as traditional medicinal plant in East-Asia. Although several studies have been conducted to the fruit composition of *R. coreanus*, research on the antioxidant composition of branch and leaves are limited. **Objectives:** We aimed to analyze the contents of five antioxidants in leaves and branches of *R. coreanus* using different extraction method. **Materials and Methods:** *R. coreanus* were cultivated at the research farm of the Gyeongsang National University. *R. coreanus* plants were harvested in May and July, and leaf and branch extracts were prepared using the ultrasonic bath and reflux extraction methods and analyzed using liquid chromatography-tandem mass spectrometry. **Results:** The ultrasonic bath extraction method extracted 7.1 and 1.5 mg/100 g gallic acid from leaves and branches, respectively, whereas the reflux extraction method yielded 12.4 and 16.5 mg/100 g, respectively. Thus, reflux extraction was superior to ultrasonic bath for both leaf and branch parts. In the extracts prepared by reflux extraction, contents of all five compounds were higher in leaves than in branches. In leaves extracted with the reflux extraction method, ellagic acid was the most abundant, followed by quercetin-3-O-glucoside (24.7 mg/100 g), gallic acid (12.4 mg/100 g), quercetin (7.3 mg/100 g), and kaempferol (1.5 mg/100 g). Contents of all compounds were higher in May (1.1 mg/g) than in July (0.7 mg/g). **Conclusion:** *R. coreanus* plant was identified to show antioxidant activity and to present abundantly five antioxidants not only in fruit but also in leaves and branches in May. Specially, quercetin was three-fold higher in leaves than in fruit juice.

Key words: Antioxidants, extract method, leaves, *Rubus coreanus*, solvent fraction

SUMMARY

- Black raspberries traditionally cultivated in Korea are *Rubus coreanus,* which has a long history of the use in traditional medicine in Korea
- Five active compounds were isolated from an aerial part (leaf, branch, and fruit) of *Rubus coreanus* include gallic acid, ellagic acid, kaempferol, quercetin, and quercetin-3-O-glucoside
- Content of all compounds was higher in leaves than branches and higher in plants harvested in May than in those harvested in July
- Contents of all compounds were higher in fruit juice than in leaves, except quercetin, which was three-fold higher in leaves than in fruit juice.



Abbreviations used: *R. coreanus: Rubus coreanus*; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; GA: Gallic acid; EA: Ellagic acid; KF: Kaempferol; QE: Quercetin; Q-3-O: Quercetin-3-O-glucoside; *m/z*: Mass-to-charge ratio.

Correspondence:

Mr. Il Rae Rho,
Institute of Agriculture & Life Science,
Gyeongsang National University, Jinju 52828,
Korea.
E-mail: irno12@gnu.ac.kr
DOI: 10.4103/pm.pm_549_19

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

The genus *Rubus* (family *Rosacea*) comprises 600–800 species distributed in temperate climate and polar regions of the world.[1-3] Fruits of *Rubus* species, commonly known as raspberries in Europe and the United States of America, are largely distinguished by their color (red, black, and purple). Approximately 20 *Rubus* species are naturally found in Korea, including *Rubus paroifolius*, *Rubus crataegifolius*, *Rubus corchorifolius*, *Rubus oldhamii* and *Rubus coreanus* ("Bokbunja," a type of black raspberry).[4]

Black raspberries conventionally cultivated in Korea are *R. coreanus*, However, the majority of products labeled as *R. coreanus* in Korea are actually *Rubus occidentalis*, which was introduced in Korea in the late 1960s. Initially, the cultivation of *R. occidentalis* started around the Jeonbuk Gochang province but later spread across the country. On the other hand, *R. coreanus* is cultivated only in a few areas in Korea.[1]

Korean black raspberry (*R. coreanus*) is distributed in Southeast Asia, particularly in Korea, Japan, and China.[5] Flowers of *R. coreanus* are

Cite this article as: Lee MJ, Nam JH, Jeong JH, Rho IR. Effect of plant part, extraction method, and harvest time over antioxidant yield of *rubus coreanus*. Phcog Mag 2020;16:S455-61.

produced at the tips of branch and bloom in May and July. These flowers are bright red in color and possess hair and the size of the petals is smaller than that of the calyx. Fruit of *R. coreanus* changed color from red to black in the early on hemispherical type as juice fruit with hairs. *R. coreanus* fruits are beneficial for human health because they exhibit antioxidative,[1,6] antipyretic,[2] anti-inflammatory,[7,8] anticancer,[9] and anti-high cholesterol.[10]

R. coreanus is rich in antioxidant compounds such as polyphenols, gallic acid, tannins, phenolic acids, organic acids (isoquercitrin), triterpenoids, flavonoids, gallotannin, ellagitannin, and anthocyanins.[11,12] Leaves and branches of *R. coreanus* plants contain tannins and flavonoids,[13] whereas fruits contain cyanidin-3-rutinoside, antocyanindins (cyandiin and pelargonidin), gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, salicylic acid, caffeic acid, p-coumaric acid, ferulic acid, m-coumaric acid, cinnamic acid, epicatechin, and protocatechuic acid.[1,14,15] The contents of antioxidant compounds were increased in the order of stem, fruit, and leaf, also the results of blood pressure improvement effect were also higher in the order of stem, fruit, and leaf.[16] Extensive research has been conducted on *R. coreanus* fruits to determine their biochemical composition; however, similar research on the leaves and branches of this species is limited.

Previously, fruit and leaves extracts of *R. coreanus* have been prepared mainly using conventional reflux heating or sonication extraction.[1,17,18] Therefore, in this study, we compared both these methods and analyzed the contents of antioxidant compounds by harvest period (May and July) in the leaves and branches of *R. coreanus*.

MATERIALS AND METHODS

Plant material

R. coreanus (IT233474) were cultivated at the research farm of the Gyeongsang National University to receive from National Agrobiodiversity Center, Republic of Korea. Leaves and branches of *R. coreanus* plants were harvested in May and July of 2018. All samples were washed, dried in shade and ground to a fine power using a mill. Then, the sample powder (10 g dry weight) was extracted by reflux and ultrasonic bath extraction methods using 100 ml of 70% methanol. In the reflux extraction method, the ground powder was extracted using the soxhelt extractor (EAM9203-06, Mtops, Yangju, Korea) for 3 h and the extracts was then filtered and concentrated using a rotary evaporator (R-520, Ilsin, Daejeon, Korea) under vacuum. The residue for fraction extraction after reflux extraction was dissolved in 60 ml of distilled water and extracted three times with an equal volume of hexane, as described previously.[19] The hexane layer was removed and an equal volume of ethyl acetate as added to the aqueous layer. Then, n-butanol and aqueous layers were processed using the same method. In the ultrasonic bath extraction method, samples were extracted with 70% methanol using ultrasonic bath (JAC-3010 (40 kHz, 200 W), Kodo, Hwaseong, Korea) at 64°C for 2 h.

All extractions were performed in triplicate. The obtained residues were evaporated under vacuum and lyophilized for 48 h in a lyophilizer (FD8508, Ilshin Biobase, Dongducheon, Korea). All freeze-dried powders were diluted 100-fold using high-performance liquid chromatography (HPLC) grade water. The samples were then filtered using a 0.22 syringe filter (Sartorius stedim biotech, Goettingen, Germany) and analyzed using liquid chromatography -tandem mass spectrometry (LC-MS/MS). Juice extracted from 20 berries was diluted 100-fold, filtered through a 0.22 syringe filer and analyzed by LC-MS/MS.

Liquid chromatography-tandem mass spectrometry analysis

LC-MS/MS analysis of *R. coreanus* leaves and branches extracts and fruit juice was performed using an HPLC system (Agilent 1100, Agilent Technologies, CA, USA) coupled to a QTRAP mass spectrometer (AB Sciex CO, CA, USA) equipped with an electrospray ionization (ESI) source to determine the contents of gallic acid, ellagic acid, kaempferol, quercetin, and quercetin-3-O-glucoside (Q-3-O).

LC analysis was performed on a YMC-Pack Pro C_{18} RS Column (150 mm × 2.0 mm × I. D., 5 μm YMC Korea Co., Ltd., SeongNam, Korea). The mobile phase consisted of acetonitrile (solvent A; Daejung Chemicals and metals Co., Ltd., Siheung, Korea): H_2O (solvent B; 0.1% formic acid), and samples were eluted with a gradient elution of 0–7 min (90% B), 7–8 min (70% B), 8–9 min (50% B), 9–10 min (20% B), 10–11 min (0% B), 11–12 min (30% B), 12–13 min (50% B), 13–14 min (70% B), and 14–45 min (100% B). The flow rate and injection volume were 0.2 ml/min and 10 μl, respectively, and the column temperature was maintained at 40°C. Gallic acid was analyzed using the same mobile phase, injection volume (10 μl), and column temperature (40°C), but with a different gradient (50% B for 0–5 min, 40% B for 5–8 min, 100% B for 8–30 min and flow rate (0.15 ml/min).

The mass spectrometer was operated under positive ion and selected ion monitoring modes. ESI was conducted using a spray voltage of 4.5 kV. The capillary voltage and the tube lens offset were fixed at −40 and −130 V, respectively. The heated capillary temperature was fixed at 350°C. Nitrogen used as the sheath and the auxiliary gas was set at 35 and 5 arbitrary units, respectively. Each component was analyzed using multiple reaction monitoring using a triple quadrupole mass spectrometer.

The mass spectra of gallic acid, ellagic acid, kaempferol, quercetin and Q-3-O were determined at mass to charge (*m/z*) ratios of 171.1, 303.2, 287.0, 303.1, and 465.3, respectively [Table 1]. Ellagic acid hydrate (Alfa Aesar, Ward Hill, MA), gallic acid (Sigma Aldrich, St. Louis, MO, USA), kaempferol (Sigma Aldrich, St. Louis, MO, USA), quercetin (Sigma Aldrich, St. Louis, MO, USA), and Q-3-O (Sigma Aldrich, St. Louis, MO, USA) were used as standards. Standard curves were prepared using serial dilutions of standards (10, 20, and 30 ppm of gallic acid and 0.1, 1, and 10 ppm of all other compounds).

Antioxidant activity test of leaves and branches in *Rubus coreanus*

Stock solutions of (1, 1-diphenyl-2-picrylhydrazyl [DPPH], Sigma Aldrich, St. Louis, MO, USA) were prepared in methanol. Stock

Table 1: Compounds detected in *Rubus coreanus* by liquid chromatography–tandem mass spectrometry analysis in positive ion modes

Compound	Formula (molecular weight)	Retention time (min)	$(M+H)^+$ *m/z*	MS/MS Fragment ion (*m/z*)	Reference fragment ions
Gallic acid	$C_7H_6O_5$ (170.1)	6.72	171.14	126.90, 125.00, 153.00, 109.00, 80.90	[42] 127.0
Ellagic acid	$C_{14}H_6O_5$ (302.2)	14.10	303.04	275.00, 264.90, 256.90, 229.10, 200.80	[43] 257.0, 275.0, 303.0
Kaempferol	$C_{13}H_{10}O_6$ (286.2)	14.39	287.03	153.00, 165.00, 137.00, 69.10	[44] 153.0, 121.0, 165.0
Quercetin	$C_{15}H_{10}O_7$ (302.2)	14.03	303.05	229.20, 164.90, 153.10, 69.10	[44] 153.0, 229.0, 137.0
Q-3-O	$C_{20}H_{20}O_{12}$ (464.3)	14.30	465.04	303.20	[45] 303.0

Q-3-O: Quercetin-3-O-glucoside; MS: Mass spectrometry

solutions of DPPH and extraction were mixed 0.8 ml of 0.4 mM and 0.2 ml, respectively. The reaction tubes were wrapped in aluminum foil and kept at room temperature for 30 min in dark. Spectrophotometric measurements were done at 517 nm using spectrophotometer (EZ Read 2000, Biochrome, Cambridge, England).

Stock solutions of ABTS (2,2'-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid, Sigma Aldrich, St. Louis, MO, USA) were prepared in water. Stock solutions of ABTS and potassium persulfate were mixed 5 ml of 7 mM and 88 µl of 140 mM, respectively. The reaction tubes were wrapped in aluminum foil and kept at room temperature for 14 h in dark. And then, dilution was performed with MeOH to give an absorbance of 0.7 approximately. Stock solutions of ABTS and extraction were mixed 1 ml and 10 µl of 140 mM, respectively. The reaction tubes were wrapped in aluminum foil and shaking for 6 min in dark. All measurements were performed under dim light. Spectrophotometric measurements were performed at 734 nm using spectrophotometer. L-ascorbic acid used as a control. DPPH and ABTS free radical scavenging activity (%) = (1-[sample absorbance/control absorbance]) ×100.

Statistical analysis

Data were analyzed using one-way analysis of variance using the SPSS program (SPSS version 21, SPSS Inc., Chicago, IL, USA). Statistical significance of the differences between mean values were assessed using Duncan's multiple range test at $P = 0.05$.

RESULTS AND DISCUSSION

Analysis method of antioxidants in leaves, branches and fruits juice of *R. coreanus*

Gallic acid produced a protonated ion (M + H) + at *m/z* 171.14 and fragment ions at *m/z* 126.90, 125.00, 153.00, 109.00, and 80.90. The corresponding *m/z* ratios of the remaining compounds were as follows: Ellagic acid, *m/z* 303.04 → 275.00, 264.90, 256.90, 229.10, and 200.80; kaempferol, *m/z* 287.03 → 153.00, 165.00, 137.00, and 69.10; quercetin, *m/z* 303.05 → 53z. 20, 164.90, 153.10, and 69.10; and Q-3-O, *m/z* 465.04 → 45z. 20 [Table 1]. Among the characteristic product ions, predomination ions at *m/z* 126.90, 275.00, 153.00, 229.20, and 303.20 were determined from gallic acid, ellagic acid, kaempferol, quercetin, and Q-3-O, respectively. The mass spectra and retention times of these compounds were in agreement with those of standards [Figure 1].

The results of this study are consistent with those of a previous study, however, fragment ions in MS^2 of gallic acid, quercetin and Q-3-O showed slight differences. Li *et al.*[20] reported that Q-3-O in *Rubus idaeus* produced a deprotonated ion at *m/z* 463.00 and in the MS/MS analysis, characteristic product ions of Q-3-O were obtained at *m/z* 300.00 (100), 301.00 (61) and 343.00 (2) with CE = 30 eV and at *m/z* 271.00, 255.00, 179.00 and 151.00 with CE = 70 eV. This suggests that the *m/z* values of fragment ions vary with the CE. In this study, we analyzed the *m/z* values of fragment ions at CE of 11, 27, 31, 37, and 23 eV for gallic acid, ellagic acid, kaempferol, quercetin, and Q-3-O, respectively.

Content of five antioxidants in leaves, branches and fruits juice of *Rubus coreanus*

In plants, gallic aicd existed as a free acid, esters, catechin derivative, and hydrolysable tannins.[21] Gallic acid and its derivatives are produced by acid hydrolysis, alkaline hydrolysis, fermentation, and enzymatic hydrolysis of tannins.[22,23] Previously, gallic acid has been extracted from *Eucalypus camaldulensis* using methanol at temperatures ranging from 25°C to 60°C or using 50% methanol at 40°C for 24 h and from *Cornus officinallis* by the hydrolysis of tannins using hydrochloric acid or heat.[24,25] Therefore, in this study, we compared the results of reflux extraction and ultrasonic bath extraction methods using methanol at 60°C. *R. coreanus* extraction yield is about 16%–18% in all treatments. The ultrasonic bath extraction method extracted 7.1 and 1.5 mg/100 g gallic acid from

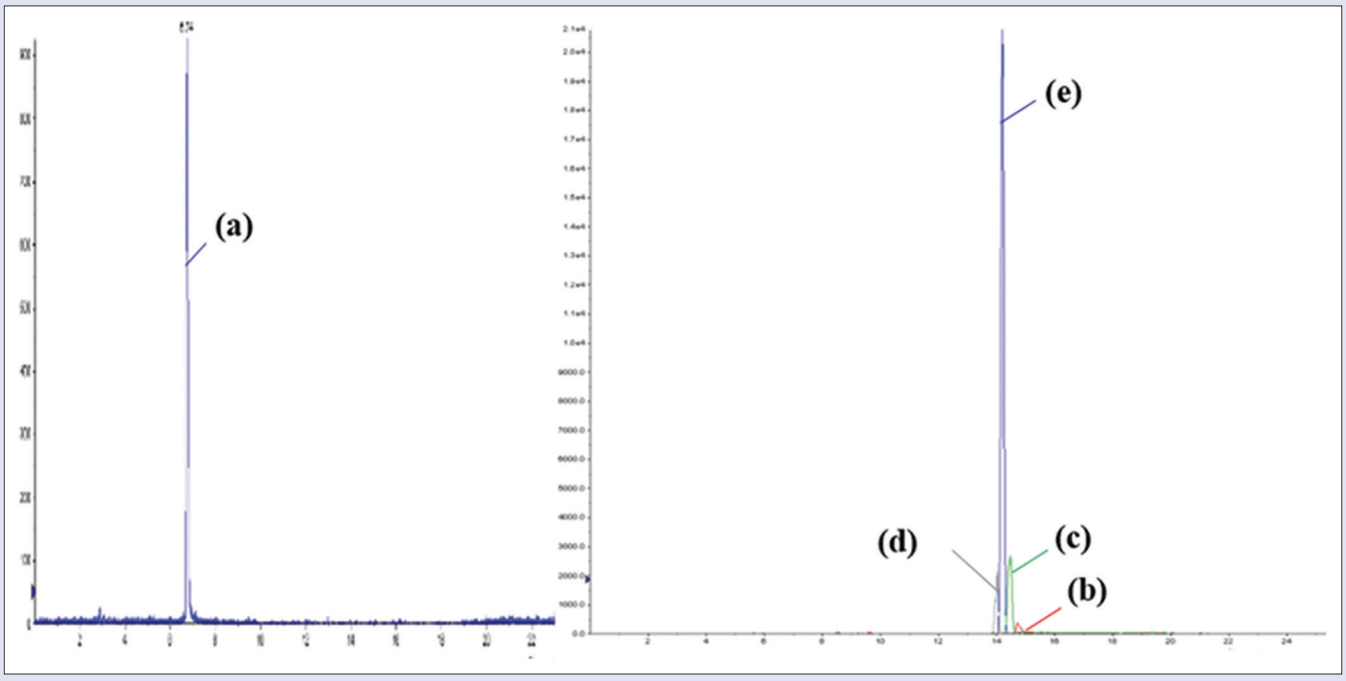


Figure 1: Liquid chromatography-tandem mass spectrometry base peak chromatograms of gallic acid (a), ellagic acid (b), kaempferol (c), quercetin (d), and quercetin-3-O-glucoside (e) obtained from extract of *Rubus coreanus* in the positive ion mode

leaves and branches, respectively, whereas the reflux extraction method yielded 12.4 and 16.5 mg/100 g, respectively. This indicates that reflux extraction is superior to ultrasonic bath extraction and gallic acid is present not only in leaves but also in branches of *R. coreanus*, although the content of gallic acid in leaves and branches was lower than that in the fruits of *R. coreanus* [Table 2 and Figure 2]. According to the previous studies, the content of gallic acid in fruit juice of *R. coreanus* varies from 0.012-0.63 mg/g.[7,26,27]

Ellagic acid exists in the free form or as a glycoside or glucose-linked ellagitannin in plant.[28,29] Therefore, ellagic acid is used to analyze hydrolyzed ellagitannins in an acidic or in temperature control.[30,31]

In this study, the amount of ellagic acid (free form) extracted from leaves and branches using the reflux extraction method (52.9 and 30.6 mg/100 g, respectively) was approximately 1.5-fold higher than that extracted using ultrasonic bath method (31.0 and 16.9 mg/g, respectively) [Table 2 and Figure 2]. In this study, ellagic acid content of *R. coreanus* fruit juice was approximately 3-fold higher than that of leaves and branches using the reflux extraction method.

According to the previous study, the content of ellagic acid in fruit juice and dried fruit were reported 1.85 and 10.6-51.5 mg/100 g for *R. coreanus* and 1.5-2 and 0.4 mg/g for *R. occidentalis*, respectively.[5,32-34] Thus, the content of ellagic acid varies according to *Rubus* species and the extraction methods. Choi *et al.*[35] reported that ellagic acid was the most abundant among 11 compounds in *R. occidentalis* fruits. Chae *et al.*[36] chose ellagic acid as the index substance in *R. coreanus* fruits. In the current study, ellagic acid was the most abundant among five compounds in leaves, branche, and fruits of *R. coreanus.*

Kaempferol and quercetin are naturally occurring plant flavonoids known for their health promoting effects. These compounds have been investigated for their pharmacological and nutraceutical activies. *R. coreanus*, blackberry, cranberry, mulberry, raspberry, strawberry, and wild raspberry reportedly contain high levels of kaempferol, quercetin, and ellagic acid.[5]

In this study, 1.5 and 0.2 mg/100 g kaempferol was extracted from the leaves and branches of *R. coreanus*, respectively, using the reflux extraction method and 0.8 and 0.5 mg/100 g was extracted using the ultrasonic bath extraction method, respectively. The content of quercetin in leaves and branches was determined as 7.3 and 6.2 mg/100 g, respectively, using reflux extraction and 5.5 and 2.7 mg/g, respectively, using ultrasonic bath. Therefore, the reflux extraction method was more effective in the isolation of kaempferol and quercetin than ultrasonic bath. Contents of kaempferol and quercetin in fruit juice were determined as 1.5 and 2.3 mg/100 g, respectively. Thus, the content of quercetin in leaves and branches was higher than that in fruit juice. Yang and Choi[5] reported the contents of kaempferol and quercetin in *R. coreanus* fruits as 0.025 and 0.470 mg/g, respectively. Thus, the kaempferol content of leaves and branches (determined using the reflux extraction method) and fruit juice in *R. coreanus* was similar to that of *R. coreanus* fruits reported previously; however, the content of quercetin determined in this study was lower than that determined previously. Yoon *et al.*[37] reported that the quercetin content of *R. coreanus* fruits was 0.25 mg/100 g, which

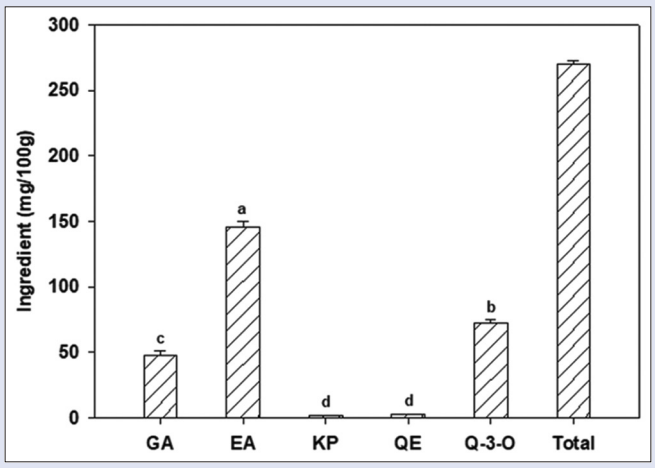


Figure 2: Comparison of ingredient contents in *Rubus coreanus* fruit. GA: Gallic acid, EA: Ellagic acid, KF: Kaempferol; QE: Quercetin; Q-3-O: Quercetin-3-O-glucoside. Vertical bars represent standard error of the means. a,b,c,d denote significant differences at $P < 0.05$

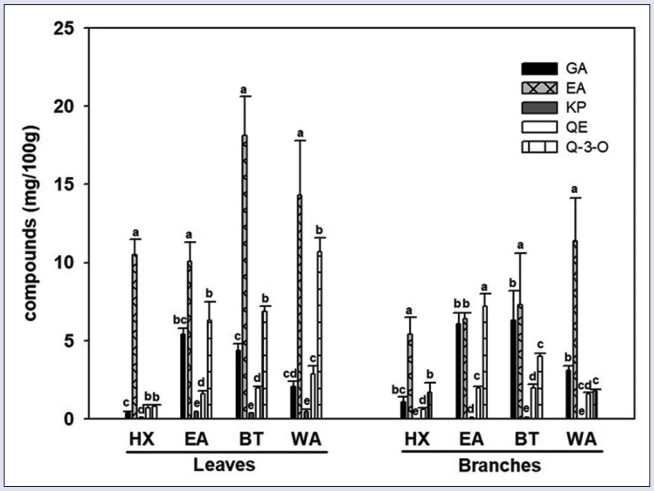


Figure 3: Comparison of ingredient contents in MeOH reflux extracts of *Rubus coreanus* according to fraction layer. GA: Gallic acid; EA: Ellagic acid; KF: Kaempferol; QE: Quercetin; Q-3-O: Quercetin-3-O-glucoside; HX: Hexane; EA: Ethyle acetate; BT: N-Buthanol; WA: Water. Vertical bars represent standard error of the means. a,b,c,d denote significant differences at $P < 0.05$

Table 2: Amounts of various compounds extracted from *Rubus coreanus* leaves and branches using reflux and ultrasonic bath extraction methods

Extraction method	Gallic acid (mg/100g)	Ellagic acid (mg/100g)	Kaempferol (mg/100g)	Quercetin (mg/100g)	Q-3-O (mg/100g)
Reflux					
Leaves	12.4±1.3^{a,b}	52.9±8.3^{a}	1.5±0.2^{a}	7.3±1.0^{a}	24.7±2.5^{a}
Branches	16.5±3.1^{a}	30.6±7.5^{b}	0.2±0.0^{c}	6.2±0.5^{a,b}	14.7±1.7^{b}
Ultrasonic bath					
Leaves	7.1±1.6^{c}	31.0±8.3^{b}	0.8±0.2^{b}	5.5±1.9^{a,b}	5.0±0.8^{d}
Branches	1.5±0.8^{d}	16.9±2.2^{c}	0.1±0.0^{d}	2.7±0.3^{c}	7.4±0.8^{c}

Data are represent means±SE. Different lowercase letters indicate significant differences at $P<0.05$. Q-3-O: Quercetin-3-O-glucoside; SE: Standard error

was approximately 10-fold higher than that of *R. idaeus* L and decrease approximately 60% degree than that of *R. idaeus* ottawa. These data suggests that the contents of kaempferol and quercetin vary according to the *Rubus* species.

The content of Q-3-O in leaves and branches of *R. coreanus* was determined 24.7 and 14.7 mg/100 g, respectively, using reflux extraction and as 5.0 and 7.4 mg/g, respectively, using ultrasonic bath, indicating that reflux extraction was more effective than ultrasonic bath. The Q-3-O content of *R. coreanus* fruits juice was 72.7 mg/100 g, which

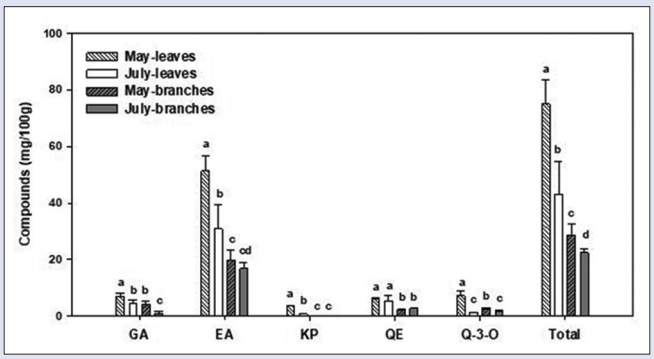


Figure 4: Comparison of ingredient contents on plant parts of *Rubus coreanus* by harvest time. GA: Gallic acid; EA: Ellagic acid; KF: Kaempferol; QE: Quercetin; Q-3-Oside: Quercetin-3-O-glucoside. Vertical bars represent standard error of the means. a,b,c,d denote significant differences at $P < 0.05$

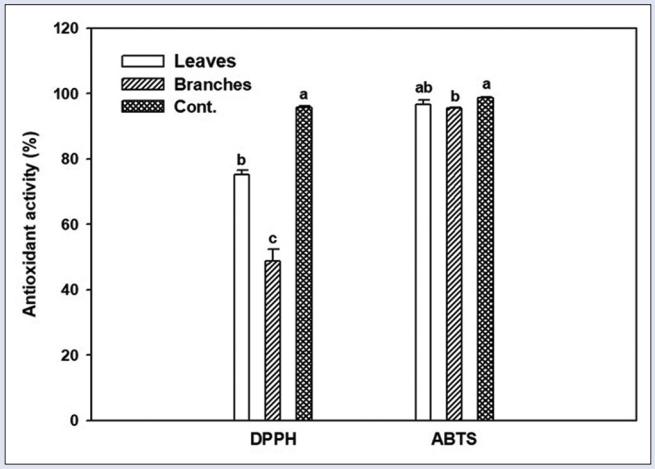


Figure 5: Comparison of DPPH and ABTS radical scavenging activity in the extracts of the leaves and branches of *Rubus coreanus* harvested in May by reflux extraction method. Vertical bars represent standard error of the means. a,b,c denote significant differences at $P < 0.05$

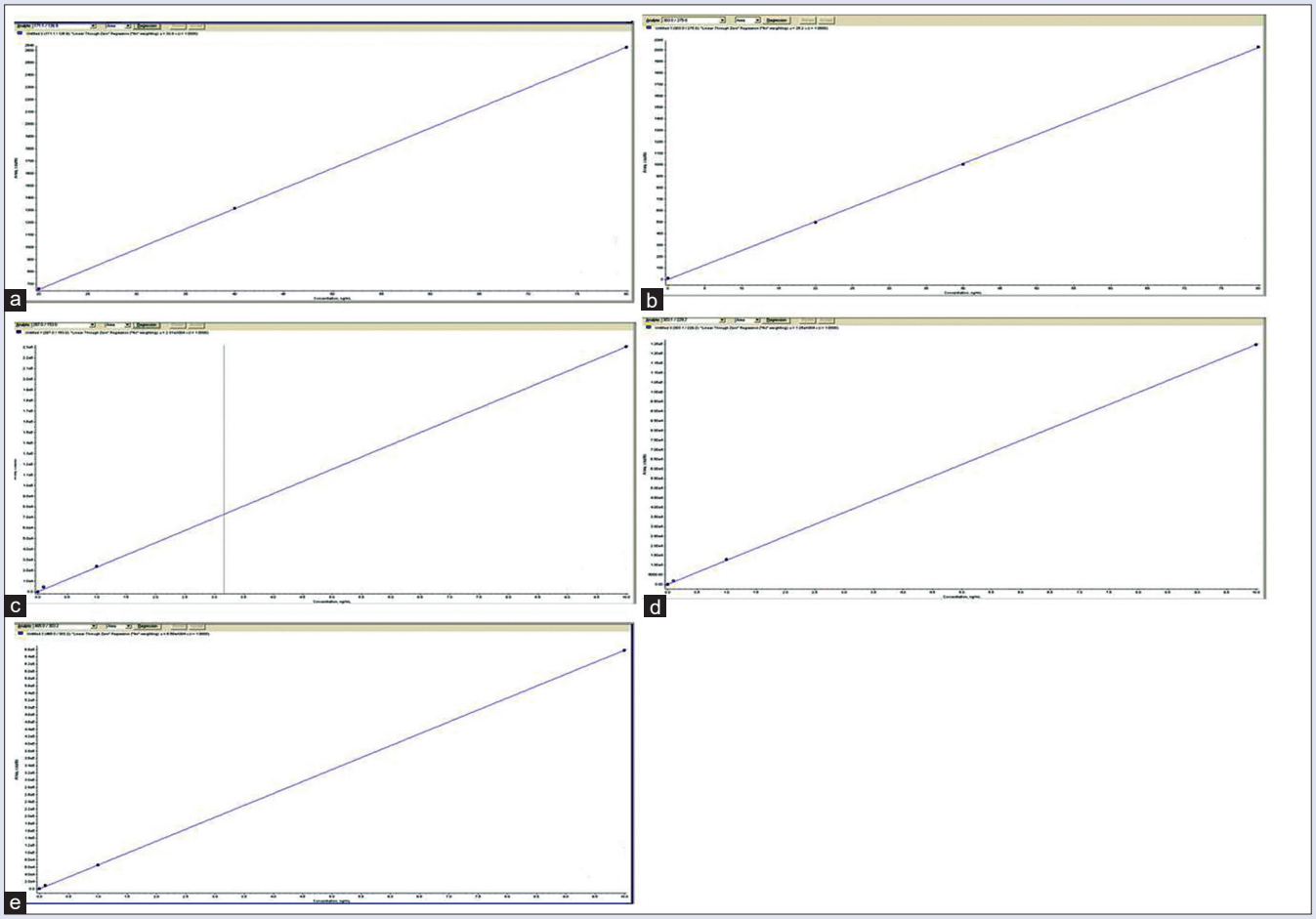


Figure 6: Liquid chromatography-tandem mass spectrometry standard curves of gallic acid (a), ellagic acid (b), kaempferol (c), quercetin (d), and quercetin-3-O-glucoside (e)

was approximately 3-fold higher than that of leaves using reflux extraction [Table 2 and Figure 2].

Li *et al.*[20] reported the protonated ion $[M + H]^-$ of Q-3-O at *m/z* 463 and fragment ions at *m/z* 417.9 and 151.0. However, these results could not be compared with the current study because the content of Q-3-O did not show. Bradish *et al.*[38] reported that the content of Q-3-O in dried fruits of *R. idaeus* varied from 1.4-3.1 mg/100 g among three cultivars including "Autumn britten," "Caroline" and "Nantahala." This suggests that the content of Q-3-O in *R. coreanus* species (leaves, branches, and fruits) is much higher than that in *R. idaeus* species, although it varied with the environment and *Rubus* species.

Comparison of extraction efficiency by solvent fraction

Solvent fraction for increasing extraction efficiency was performed after methanol-based reflux extraction from *R. coreanus* leaves and branches. The content of ellagic acid was relatively higher in the n-butanol and aqueous layer, although it was distributed evenly in all layers. Q-3-O was distributed in all layers except the hexane layer. Although the content of gallic acid in leaves and branches extracts was relatively higher in the ethyl acetate layer of low polarity, it was also present in the n-butanol and aqueous layers. Kaempferol and quercetin were evenly distributed in all of layers [Figure 3]. These data suggest that to prepare the leaves and branches extracts of *R. coreanus*, it is more desirable to use the methanol-based reflux extraction than the solvent fraction because compounds identified in this study were distributed in all layers.

Our results showed that the content of all antioxidant compounds was higher in leaves than branches and higher in plants harvested in May than in those harvested in July [Figure 4]. It was difficult to harvest *R. coreanus* plants because of the presence of thorny. Therefore, when using the extract of the leave and branches of *R. coreanus*, it is advisable to harvest branches with attached leaves in May because the active compounds are present in both leaves and branches.

Antioxidant activity test in leaves and branches of *Rubus coreanus*

Antioxidant activity in leaves and branches of *R. coreanus* were determined using both DPPH and ABTS radical scavenging method. The DPPH activity was 95.8% in control, 48.8% in branches, and 75.1% in leaves of *R. coreanus*. Hence, leaves are about 26.3% higher than branches and show significant differences. ABTS activity was 98.7% in control, 95.6% in branches, and 96.6% in leaves of *R. coreanus*, which showed higher activity than branches [Figure 5]. Thus, the results showed that both DPPH and ABTS of *R. coreanus* had higher antioxidant radical activity in the leaves than in the branches.

CONCLUSION

In this study, we analyzed the contents of five antioxidant compound in *R. coreaus* leaves, branches and fruit juice by LC-MS/MS. The reflux extraction method was superior to the ultrasonic bath extraction method, as the former yielded higher contents of compounds from leaves and branches than the latter. In leaves, the content of ellagic acid was highest, followed by Q-3-O, gallic acid, quercetin, and kaempferol. Contents of all compounds were higher in fruit juice than in leaves, except quercetin, which was 3-fold higher in leaves than in fruit juice. In addition, contents of all compounds were higher in May than in July. *R. coreanus* plant was identified to show antioxidant activity and to present abundantly five antioxidants not only in fruit but also in leaves and branches in May.

Financial support and sponsorship

This research was supported by Grant No. PJ01383404 from the Agricultural R and D, Rural Development Administration, Republic of Korea.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kim LS, Youn SH, Kim JY. Comparative study on antioxidant effects of extracts from *Rubus coreanus* and *Rubus occidentalis*. J Korean Soc Food Sci Nutr 2014;43:1357-62.
2. Ali N, Shaoib M, Shah SW, Shah I, Shuaib M. Pharmacological profile of the aerial parts of *Rubus ulmifolius* Schott. BMC Complement Altern Med 2017;17:7. Available from: https://doi.org/10.1186/s12906-017-1564-z. [Last accessed on 2017 Jan 19]
3. Marulanda ML, Lopez AM, Uribe M. Genetic diversity and transferability of *Rubus* microsatellite markers to South American *Rubus* species. In: Caliskan M, editor. The Molecular Basis of Plant Genetic Diversity. London: INTECH; 2012. p. 151-64.
4. Lee MK, Lee HS, Choi GP, Oh DH, Kim JD, Yu CY, *et al.* Screening of biological activities of the extracts from *Rubus coreanus* Miq. Korean J Med Crop Sci 2003;11:5-12.
5. Yang JW, Choi IS. Comparison of the phenolic composition and antioxidant activity of Korean black raspberry, Bokbunja, (*Rubus coreanus* Miquel) with those of six other berries. Cyta J Food 2016;15:110-7.
6. Lee JE, Park EK, Lee JE, Auh JH, Choi HK, Lee JH, *et al.* Effects of a *Rubus coreanus* Miquel supplement on plasma antioxidant capacity in healthy Korean men. Nutr Res Pract 2011;5:429-34.
7. Lim JW, Hwang HJ, Shin SC. Polyphenol compounds and anti-inflammatory activities of Korean black raspberry (*Rubus coreanus* Miquel) wines produced from juice supplemented with pulp and seed. J Agric Food Chem 2012;60:5121-7.
8. Yang HM, Lim SS, Lee YS, Shin HK, Oh YS, Kim JK. Comparison of the anti-inflammatory effects of the extracts from *Rubus coreanus* and *Rubus occidentalis*. Korean J Food Sci Technol 2007;39:342-7.
9. Jung JW, Son MY, Jung SW, Nam PW, Sung JS, Lee SJ, *et al.* Antioxidant properties of Korean black raspberry wines and their apoptotic effects on cancer cells. J Sci Food Agric 2009;89:970-7.
10. Lee MJ, Choi HR, Lee JH, Lee SJ, Kwon JW, Choi KM, *et al.* Co-treatment with fermented black raspberry and red ginseng extracts improves lipid metabolism and obesity in rats fed with a high-fat and high-cholesterol diet. Korean J Food Sci Technol 2015;47:364-72.
11. Lee JM, Dossett M, Finn CE. Rubus fruit phenolic research: The good, the bad and the confusing. Food Chem 2012;130:785-96.
12. Ju HK, Cho EJ, Jang MH, Lee YY, Hong SS, Park JH, *et al.* Characterization of increased phenolic compounds from fermented bokbunja (*Rubus coreanus* Miq.) and related antioxidant activity. J Pharm Biomed Anal 2009;49:820-7.
13. Lee YA, Lee MW. Tannins from *Rubus coreanus*. Korean J Pharmacogn 1995;26:27-30.
14. Kim HS, Park SJ, Hyun SH, Yang SO, Lee JH, Auh JH *et al.* Biochemical monitoring of black raspberry (*Rubus coreanus* Miquel) fruits according to maturation stage by 1H-NMR using multiple solvent systems. Food Res Int 2011;44:1977-87.
15. Deighton N, Brennan R, Finn C, Davis HV. Antioxidant properties of domesticated and wild *Rubus* species. J Sci Food Agric 2000;80:1307-13.
16. Lee MJ, Lee SJ, Choi HR, Lee JH, Kwon JW, Lee TB, *et al.* Improvement of cholesterol and blood pressure in fruit, leaf and stem extracts from black raspberry *in vitro*. Korean J Med Crop Sci 2014;22:177-87.
17. Hui T, Lee WY, Choi YH. Optimization of microwave-assisted extraction for anthocyanins, polyphenols, and antioxidants from raspberry (*Rubus coreanus* Miq.) using response surface. J Sep Sci 2013;36:3107-14.
18. 18Hyun TK, Rim YG, Kim EY, Kim JS. Variation in bioactive principles of Korean black raspberry (*Rubus coreanus* Miquel) during ripening. Plant Omics 2014;7:510-6
19. 19Shibata ST, Ando T, Tanaka O. Chemical studies on oriental plant drugs. XIV. Protopanaxadiol, sapogenin of the ginseng saponin. Chem Pharm Bull 1966;14:595-600.
20. Li ZH, Guo H, Xu WB, Ge J, Li X, Alimu M, *et al.* Rapid identification of flavonoid constituents directly from PTP1B inhibitive extract of raspberry (*Rubus idaeus* L.) leaves by HPLC–ESI–QTOF–MS-MS. J Chromatogr Sci 2016;54:805-10.

21. Karamac M, Kosinska A, Pegg RB. Content of Gallic acid in selected plant extracts. Pol J Food Nutr Sci 2006;15:55-8.
22. Mammela P, Tuomainen A, Savolainen H, Kangas J, Vartiainen T, Lindroos L. determination of Gallic acid in wood dust as an indicator of Oak content. J En Viron Monit 2001;3:509-11.
23. Wang XH, Cai C, Li XM. Optimal extraction of Gallic acid from *Suaeda glauca* Bge. Leaves and enhanced efficiency by ionic liquids. Int J Chem Eng Appl 2016;2016:166-74.
24. Daneshfar A, Ghaziaskar HS, Homayoun N. Solubility of Gallic acid in methanol, ethanol, water and ethyl acetate. J Chem Eng 2008;53:776-8.
25. Zhang L, Wang YM, Xu M. Quantification of Gallic Acid and Ellagic Acid from the Seed of *Cornus officinalis* by UHPLC method and their antioxidant activity. Chem Eng Commun 2014;201:545-56.
26. Adina F, Cecilia G, Felicia G, Carmen D, Ovidiu T. Identification and quantification of phenolic compounds from red currant (*Ribes rubrum* L.) and raspberries (*Rubus idaeus* L.). Int J Pharmacol Phytochem and Ethnomed 2017;6:30-7.
27. Teng H, Lee WY, Choi YH. Optimization of microwave-assisted extraction for anthocyanins, polyphenols and antioxidants from raspberry (*Rubus coreanus* Miq.) using response surface Methodology. J Sep Sci 2013;36:3107-14.
28. Abe LT, Lajolo FM, Genovese MI. Potential dietary sources of Ellagic acid and other antioxidants among fruits consumed in Brazil: Jabuticaba (*Myrciaria jaboticaba* (Vell.) Berg). J Sci Food Agric 2012;92:1679-87.
29. Koponen JM, Happonen AM, Mattila PH, Törrönen AR. Contents of anthocyanins and ellagitannins in selected foods consumed in Finland. J Agric Food Chem 2007;55:1612-9.
30. Kool MM, Comeskey D, Cooney JM, McGjie TK. Structural identification of the main ellagitannins of a boysenberry (*Rubus loganbaccus baileyanus* Britt.) extract by LC–ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy. Food Chem 2010;119:1535-43.
31. Mullen W, Yokotab T, Lean ME, Crozier A. Analysis of ellagitannins and conjugates of Ellagic acid and quercetin in raspberry fruits by LC–MS. Phytochemistry 2003;64:617-24.
32. Djurić M, Maškovic P, Murtić S, Veljković B, Ćurčić S, Paunović G, *et al*. Quantitation of Ellagic acid in blackberries. Hem Ind 2014;68:241-5.
33. Gundogdu M, Kan T, Canan I. Bioactive and antioxidant characteristics of blackberry cultivars from East Anatolia. Turk J Agric For 2016;40:344-51.
34. Daniel EM, Krupnick AS, Geur YH, Blinzler J, Nims RW, Stoner GD. Extraction, stability and quantitation of Ellagic acid in various fruits and nuts. J Food compos Anal 1989;2:338-49.
35. Choi HR, Lee SJ, Lee JH, Kwon JW, Lee HK, Jeong JT, *et al*. Cholesterol-lowering effects of unripe black raspberry water extract. J Korean Soc Food Sci Nutr. 2013;42:1899-907.
36. Chae KS, Son RH, Park SY, Kim KA, Lee TB, Kwon JW. Analytical method validation of Ellagic acid as a marker compound for the standardization of black raspberry extract as a functional ingredient. Food Eng Prog 2014;18:355-8.
37. Yoon I, Hyang WJ, Hak MJ, Ahn TH, Park KH. Isolation and identification of quercetin with antioxidative activity from the fruits of *Rubus coreanus* Miquel. Korean J Food Sci Technol 2003;35:449-502.
38. Bradish CM, Perkins-Veazie P, Fernandez GE, Xie G, Jia W. Comparison of flavonoid composition of red raspberries (*Rubus idaeus* L.) grown in the Southern United States. J Agric Food Chem 2012;60:5779-86.