

Original Research Article

Evaluation of antioxidant and anti-inflammatory effects of three different *Rubus coreanus* Miq. by-products

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Sent for review: 11 May 2021

Revised accepted: 3 October 2021

Abstract

Purpose: To investigate the antioxidant and anti-inflammatory properties of three different *Rubus coreanus* Miq. by-products in stimulated BV-2 microglial cells and explore its underlying physiological efficacy.

Methods: Cell viability assessment was performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Lipopolysaccharide (LPS) was used to activate BV-2 microglia. Total polyphenol and total flavonoid contents were determined by the method of Folin-Denis. As three different *Rubus coreanus* Miq. by-products remaining after extraction of *Rubus coreanus*, High performance liquid chromatography (HPLC) finger printing, ABST (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, and NO (nitric oxide) inhibitory assay were performed.

Results: Three different *Rubus coreanus* by-product extract did not exhibit any signs of cytotoxicity to BV-2 cells up to 100 µg/ml concentration ($p < 0.5$). The LPS-activated excessive release of NO in BV-2 cells was significantly inhibited by *Rubus coreanus* by-product extract ($p < 0.5$) at 500 µg/mL. Total polyphenol and total flavonoid contents were highest in 50 % ethanol wine processing by-product ($p < 0.5$ at 30, 50, 70 and 100 %, respectively). The by-product of wine processing had the lowest RC_{50} radical scavenging effect (16.53 µL/ml). The quercetin content of the wine processing by-product was the highest in the 70% ethanol extract at 6.26 mg/g ($p < 0.5$ at 30, 50, 70 and 100%, respectively).

Conclusion: These results reveal that of the three other by-products, wine processing by-product has the highest antioxidant and anti-inflammatory activities. The use of these by-products has high added value for industrial production; furthermore, they are a potential treatment for various inflammatory diseases.

Keywords: *Rubus coreanus*, By-product, Anti-oxidant, Anti-inflammatory

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INTRODUCTION

Rubus coreanus Miq. is a deciduous broadleaf shrub belonging to the family *Rosaceae* that grows in to the far-east Asian countries such as

China, Japan, and South Korea [1]. In Korea, it is called 'bokbunja', and has been used for treating asthma, enuresis, spermatorrhea, and allergic diseases in oriental medicine [2]. In general, among the components of *R. coreanus* (100 g),

moisture 87.0 g, protein 1.6 g, fat 1.7 g, glucose 1.3 g, fiber 2.9 g, ash 0.5 g, etc. are variously contained. As inorganic salts, potassium (K), Calcium (Ca), magnesium (Mg), sodium (Na), iron (Fe), and the like, and vitamins contain vitamin A, vitamin B1, B2, and vitamin C [3]. In addition, it is reported to have functions such as anti-cancer activity, antioxidant activity, cholesterol-lowering effect and anti-tumor activity [4] because it contains useful compounds such as gallic acid, tannin, catechin, and quercetin [5].

Although *R. coreanus* is mainly consumed as a raw fruit, its use is increasing as a raw material for wine and juice due to an increase in preference for natural fruit juice, and recently, it has been used in various ways such as jelly and jam [6]. *Rubus c.* processing by-products are about 20% of the total *R. coreanus*, and some are used as feed, but most of them are discarded [7]. Ku et al. [7] reported that the hot water extract of *R. coreanus* processing by-products contained organic acids 8.9 %, neutral sugar 69.9 %, pectin 19.1 %, anthocyanins 5.2 %. Jeong and Seo [8] reported a study on the polyphenol content of *R. coreanus* processing by-products, but studies using *R. coreanus* processing by-products are rare. In the body, biochemical reactions constantly take place to supply the energy required for metabolism, thereby generating free radicals [9]. These free radicals are continuously produced as by-product of metabolic processes in the body, and are unstable and highly reactive [10]. When oxidative stress occurs because excessive active oxygen is generated in the body and cannot be cleared through normal metabolism [11], cells and tissues are damaged, leading to various degenerative diseases such as cancer, heart disease, arteriosclerosis, dermatitis, and dementia [12]. Herbal medicines are known to contain large amounts of antioxidants that can remove active oxygen. The aim of this study is to promote the potential utilization of *R. coreanus* processing by-products as a medicinal herb. Therefore, in this study, extracts were prepared by different extraction temperatures and ethanol ratio conditions of raw *R. coreanus* and by-products generated after processing juice and wine of *R. coreanus*, and optimal extraction conditions were established through antioxidant and anti-inflammatory screening.

EXPERIMENTAL

Preparation of *R. coreanus* extracts

Raw fruits of *R. coreanus* were collected from Gochang (Jeollabukdo, Korea) on August, 2019. Juice and wine by-products of *R. coreanus* were

obtained from a processing plant of *R. coreanus* in Gochang (Jeollabukdo, Korea). The various extraction conditions of *R. coreanus* are shown in Table 1. All extracts were extracted for 24 h.

Total polyphenol and flavonoid contents (TPC and TFC)

Total polyphenol content of *R. coreanus* extract was determined by the method of Folin-Denis [13]. After the *R. coreanus* extract was prepared at a concentration of 1 mg/mL, Folin & Ciocalteu's phenol reagent 60 μ L was added to sample solution 60 μ L. After 3 min, 60 μ L of a 10% sodium carbonate (Na_2CO_3) (Sigma Chemical Co., St. Louis, Mo. USA) was added, allowed to stand at RT for 1 h. The absorbance of mixture was read at 750 nm (Bio-Rad Inc., Hercules, CA, USA). The standard curve was used to calculate the total polyphenol content of the sample extracts which was expressed as gallic acid (Sigma Chemical Co.) equivalent mg (GAE)/g dry weight. Total flavonoid content of *R. coreanus* extract was evaluated by a colorimetric method described by Nieva Moreno et al. [14]. After diluting the *R. coreanus* extract (100 μ L) was mixed with 20 μ L of 1 M potassium acetate and 10% aluminum nitrate adding 860 μ L of 80% ethanol. After 40 min, the absorbance of the reaction mixture was then measured at 415 nm (Bio-Rad Inc.). The total flavonoid content was determined with respect to the standard curve of quercetin (Sigma Chemical Co.), and was expressed as quercetin equivalent.

ABTS radical scavenging activity

ABTS radical scavenging activity of *R. coreanus* extract was determined by the method of Re et al. [15]. $\text{ABTS}^{\cdot+}$ cation radical was produced by the reaction between 7 mM ABTS in PBS and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 24 h before use. $\text{ABTS}^{\cdot+}$ solution was then diluted with PBS to obtain an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 20 μ L of *R. coreanus* extract to 180 μ L of diluted $\text{ABTS}^{\cdot+}$ solution, the absorbance was measured at 1 min after the initial mixing. The ABTS radical scavenging activity was calculated by the following equation.

$\text{ABTS}^{\cdot+}$ scavenging effect (%) = $((\text{AB}-\text{AA})/\text{AB}) \times 100$, where, AB is absorbance of $\text{ABTS}^{\cdot+}$ radical + methanol; AA is absorbance of $\text{ABTS}^{\cdot+}$ radical + sample extract/standard.

Identification of quercetin by HPLC

The content of quercetin in *R. coreanus* extract was analyzed by the method of Yang et al [16].

The *R. coreanus* extract was dissolved in 99 % methanol to prepare solutions at 10 mg/mL. Solutions were filtered through 0.22 μ m filters. Using a 20 μ L sample loop, the sample was analyzed using a reversed-phase HPLC system (Waters 2690, MA, USA), a quaternary pump, and a vacuum degasser. Quercetin separation was performed by a prepacked ZORBAX C₁₈ column (XDB-C₁₈, 4.6 \times 150 mm, 5 μ m). Two mobile phases were used: solvent A, 5% acetic acid; B, 100% acetonitrile. The gradient for HPLC analysis was linearly changed as follow (total 50 min): 5 % B at 0 min, 25 % B at 20 min, 100 % B at 21 min, 100 % B at 35 min, 5 % B at 36 min, 5 % B at 50 min. Flow rate was set to 1.0 mL/min.

Cell culture, cytotoxicity, and nitrite measurement

BV-2 microglia cells were cultured in RPMI1640 (Gibco BRL, NY, USA) containing 10% fetal bovine serum (FBS; Gibco BRL) and 1 % antibiotic-antimycotic (Gibco BRL) at 37°C in 5% CO₂. Cell viability against *R. coreanus* extracts was measured by a 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells (1 \times 10⁵ cell / mL) were dispensed in 100 μ L in 96 well-plates and cultured in a CO₂ incubator for more than 12 h, and then followed by incubation for 24 h by treating 100 μ L of fresh medium and *R. coreanus* extract (100 and 500 μ g/mL). After 24 h, 10 μ L of MTT (2.5 mg/mL) was added, followed by incubation for 4 h. And then, the culture medium was removed, and 100 μ L of dimethylsulfoxide (DMSO) was added to dissolve the generated formazan crystals, which was measured at 540 nm using a microplate reader. Cell viability was expressed as a percentage (%) compared to the control. The inhibitory effect of nitric oxide (NO) was measured by reacting the NO₂⁻ form present in the cell culture with Griess Reagent using the method of Green *et al* [17]. BV-2 cells (1 \times 10⁵ cell/mL) were cultured in 96-well plates overnight. The cells were treated with *R. coreanus* extracts (100 and 500 μ g/mL) and 100 ng/mL of lipopolysaccharide (LPS) for 24 h. And then, 100 μ L of cell culture solution and 100 μ L of Griess reagent (Sigma Chemical Co.) were mixed and reacted in 96 well-plates for 10 min, and then measured at 540 nm using a microplate reader.

Data analysis

The results were expressed as mean \pm SD and statistically analyzed by analysis of variance (ANOVA). Duncan's multiple range test was performed to determine significant differences

among the groups, and differences at $p < 0.05$ were considered significant.

RESULTS

Total polyphenol (TPC) and total flavonoid contents (TFC)

Total phenolic and flavonoid contents of *R. coreanus* processing by-products extracted by using different concentration of ethanol and different temperature of water are presented in figure 1. The results showed that the recovery of phenolic and flavonoid compounds was significantly varied depending on the temperature of water and ratios of the ethanol used. The TPC of water temperature-based extract ranged from 35.94 mg GAE/g to 105.17 mg GAE/g, from 29.72 mg GAE/g to 242.52 mg GAE/g for the ethanol concentration-based extract, respectively. Besides, all the extracts of wine processing by-product showed the highest amounts of TPC (Fig. 1A).

The results of TFC were expressed as mg quercetin equivalent (QE) per gram dry extract. The TFC of water temperature-based extract ranged from 13.21 mg QE/g to 55.62 mg QE/g, from 10.27 mg QE/g to 72.68 mg QE/g for the ethanol concentration-based extract, respectively (Fig. 1B).

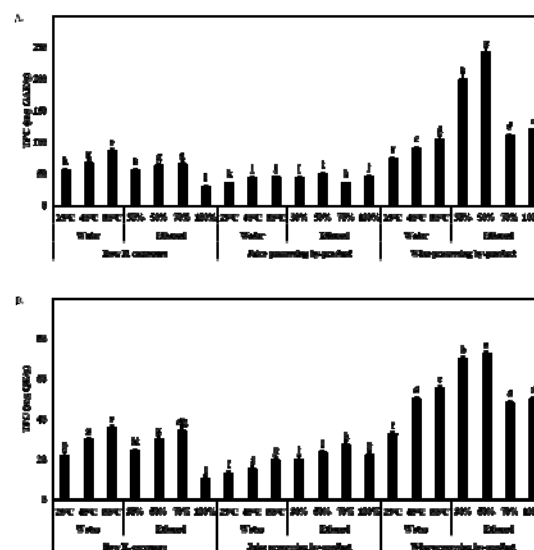


Figure 1: Total polyphenol and flavonoid content of the raw *R. coreanus* and by-products by various extraction conditions. (A) Total polyphenol content analyzed as gallic acid equivalent (GAE) mg/g of extract. (B) Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract. Different superscripts in a column indicate significant differences at $p < 0.05$ by Duncan's multiple range test

ABTS radical scavenging effect

Figure 2 shows RC₅₀ values of *R. coreanus* processing by-products extracted by using different concentration of ethanol and different temperature of water. It can be seen from the results that the wine processing by-product showed the lowest RC₅₀ value, demonstrating high antioxidant activity, followed by raw *R. coreanus* and juice processing by-products, respectively.

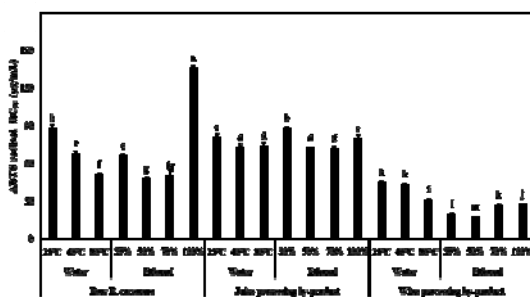


Figure 2: ABTS radical scavenging activity of the raw *R. coreanus* and by-products with various extraction conditions. RC₅₀ is amount required for 50% reduction of ABTS radical scavenging activity. Data are mean±standard deviation (n=3). Different superscripts in a column indicate significant differences at *p* < 0.05 by Duncan's multiple range test

Quercetin content

In this study, the quercetin contents of raw *R. coreanus* and by-products generated after processing juice and wine of *R. coreanus* by various extraction conditions was analyzed by HPLC analysis, and presented in figure 3. The content of quercetin was in the range of 0 - 6.26 mg/g, and the extracts of wine processing by-product was contained the highest quercetin content. Overall, the extraction method showed optimal quercetin extraction when 50% ethanol solvent was used.

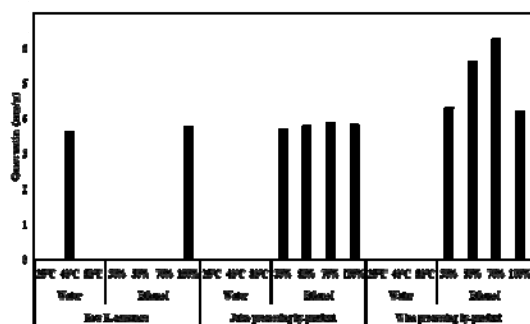


Figure 3: The quercetin contents of the raw *R. coreanus* and by-products by various extraction conditions

Cytotoxicity

To determine the cell cytotoxicity of *R. coreanus* processing by-products extract treatments, we carried out the MTT assay to measure the viability of cells cotreated with *R. coreanus* processing by-products extract/LPS (Figure 4). Based on our results, treatments of raw *R. coreanus* and juice processing by-products up to 500 µg/mL for 24 h had no cytotoxic effects in comparison with the control group. However, 70 and 100 % ethanol extract of wine processing by-product at a dose of 500 µg/mL significantly reduced the viability of BV-2 cells with LPS.

Nitric oxide (NO) inhibitory effects

We examined the anti-inflammatory effect of *R. coreanus* processing by-products extract by investigating the levels of the proinflammatory mediators NO in LPS-stimulated BV-2 cells (Figure 1.B). Our results confirmed the water extract had no NO inhibition effect, but the ethanol extract showed a NO inhibition effect ranging from 14.94 to 67.72% excluding cytotoxic concentrations. Especially, 50% ethanol extract of wine processing by-product have been proven as the best extraction condition for NO inhibition effect.

DISCUSSION

R. coreanus is well known to have various pharmacological effects, including anti-inflammatory and antioxidant [2] activities, which are attributed to the phenolic compounds such as anthocyanin, gallic, vanillic, caffeic acids, *p*-hydroxybenzoic, salicylic, syringic, and protocatechuic. [3,5]. However, the effect of *R. coreanus* processing by-products and extraction conditions on the antioxidant and anti-inflammatory effects of *R. coreanus* have not yet been examined. This is the first study to compare the antioxidant and anti-inflammatory effects of different extraction condition extracts of *R. coreanus* processing by-products.

Phenolic compounds, which are widely distributed in medicinal herbs, are well known as natural antioxidants that act as reducing agents, hydrogen or electron donors, metal cations chelators, and singlet oxygen quenchers [18]. Polyphenol compounds have an aromatic ring with two or more phenolic hydroxyl groups in the molecule, and there are more than 1,000 types such as phenolic acid derivatives, anthocyanins, flavonoids, lignans, resveratrol and tannins [19].

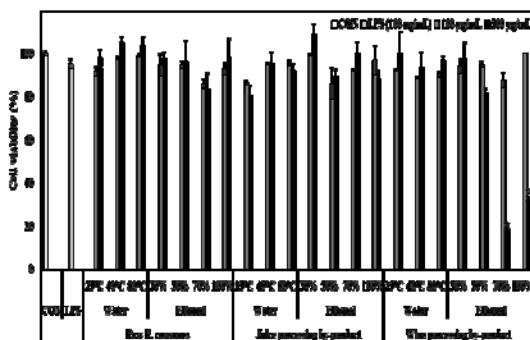


Figure 4: Effect of the raw *R. coreanus* and by-products by various extraction conditions on the BV-2 microglial cell viability. Cell viability in *R. coreanus* extract-treated cells was determined using MTT assay. All values are expressed as mean \pm SD. Different letters are significantly different at $p < 0.05$ by Duncan's multiple range test

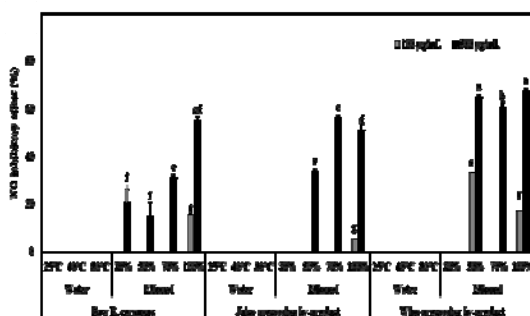


Figure 5: Effect of the raw *R. coreanus* and by-products by various extraction conditions on the BV-2 microglial cell viability. BV-2 cells were treated with different concentrations of each sample (100 and 500 $\mu\text{g/mL}$) and LPS (100 ng/mL) for 24 h. And the NO contents in supernatant was measured using Griess reagent. Data are mean \pm standard deviation ($n=3$). Different superscripts in a column indicate significant differences at $p < 0.05$ by Duncan's multiple range test

These results clearly showed that the order of the extracts with the highest level of TPC in wine processing by-product was as follows: 50 % ethanol > 30 % ethanol > 100 % ethanol > 70 % ethanol > 80 °C water > 40 °C water > 25 °C water extract. Among the kind of processing by-product, wine processing by-product extracted using 50% ethanol showed the highest amount of TFC. Similar to TPC, TPC and TFC of wine processing by-product were the highest among the processing by-product irrespective of the extraction condition used. Previously, many researchers reported that the increase in total phenolic during fermentation of natural materials was a result of enzymatic reactions such as microbial-derived β -glucosidase or esterase [20]. In this study, the increase in TPC and TFC in

wine processing by-products is thought to be a result of several enzymes generated from the metabolism of yeast used in wine processing.

In the present study, ABTS free radical-scavenging assays was used to determine the antioxidant capacity of different extraction condition extracts of *R. coreanus* processing by-products. The antioxidant activity is usually expressed either as percentage reduction of ABTS or RC_{50} , which indicates 50% inhibition of ABTS radicals. The lowest RC_{50} value shows the most potent antioxidant activity. In the present work, the scavenging activity was expressed as RC_{50} ($\mu\text{g/mL}$). Wine processing by-product showed the lowest RC_{50} value, demonstrating high antioxidant activity. This result was correlated with the highest TPC and TFC in the 50% ethanol concentration of wine processing by-product. 50 % ethanol extract of wine processing by-product have been proven as the best extraction condition for the extraction of antioxidants.

Quercetin is a flavonoid with antioxidant and anti-inflammatory properties [21]. This compound is present in fruit, vegetables, and is abundant in red wine [22]. In this study, it was confirmed that quercetin extract, a bioactive compound, was the highest when extracted with 50% ethanol from wine processing by-product of *R. coreanus* Song *et al.* [23] showed optimal extraction results in the 40 - 80% ethanol ratio of the extraction efficiency of polyphenols, flavonoids, and chlorogenic acid, which are bioactive compounds of *Lonicera japonica Flos*. Therefore, the ratio of the extraction method and solvent should be considered according to the bioactive compound to be extracted.

Among the inflammatory mediators, reactive oxygen species, nitric oxide (NO) in particular, initiates a wide range of toxic oxidative reactions causing tissue injury [24]. Our results clearly showed that *R. coreanus* ethanol extract attenuated NO production. Similar to the antioxidant results, 50% ethanol extract of wine processing by-product was the most attenuated NO production without cytotoxicity. This result indicates that 50 % ethanol extract of wine processing by-product acts principally by regulating NO generation and could be beneficial for preventing the progression of neuro-inflammation by BV-2 microglial activation.

CONCLUSION

The findings of this study show that wine processing by-product of *R. coreanus* inhibits inflammatory responses in LPS-stimulated BV-2

microglial cells and has the potential to be developed into a therapeutic agent for the treatment of various inflammatory diseases.

DECLARATIONS

Acknowledgement

The present research was supported by the research fund of Dankook University in 2020.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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