

Anti-Inflammatory and Anti-Superbacterial Activity of Polyphenols Isolated from Black Raspberry

Seong Keun Kim¹, Hyuna Kim², Song Ah Kim¹, Hee Kuk Park¹, and Wonyong Kim¹

¹Department of Microbiology, College of Medicine, ²Department of Economics, Faculty of Business and Economics, Chung-Ang University, Seoul 156-756, Korea

The fruit of the black raspberry (*Rubus coreanus* Miquel) has been employed in traditional medicine, and recent studies have demonstrated its measurable biological activities. However, the root of the black raspberry has not been studied. Therefore, in this study, we evaluated the anti-inflammatory and antibacterial properties of the root and unripe fruit polyphenols of the black raspberry. Both polyphenols proved to have anti-inflammatory activity as evidenced by the decreased nitric oxide (NO), cytokines (IL-1 β , IL-6, and IL-10) and prostaglandin E₂ (PGE₂) levels in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages. However, root polyphenols showed stronger anti-inflammatory activity than fruit polyphenols. LPS-induced mRNA and protein expressions of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 levels were also decreased, confirming the anti-inflammatory activity. Root polyphenols showed lethal activity against methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* (CRAB), and *Bacillus anthracis*. In contrast, the black raspberry fruit did not demonstrate these properties. These data provide the first demonstration that black raspberry root has potential anti-inflammatory and anti-superbacterial properties that can be exploited as alternatives for use in the food and cosmetic industries and/or as pharmaceuticals.

Key Words: Anti-inflammatory effect, Antimicrobial activity, Black raspberry, Cytokine, RAW 264.7 murine macrophages

INTRODUCTION

Black raspberry is a perennial shrub belonging to the *Rosaceae* family that grows wild in Far East Asian Countries, namely South Korea, Japan and China [1]. The fruit, a type of black raspberry called “Bokbunja” in Korean, has long been used in traditional alternative medicine for liver and kidney diseases, spermatorrhoea and prostate and urinary diseases [2,3].

Inflammation is inherent to the pathogenesis of a variety of diseases. Macrophages are critical immune cells in the regulation of inflammatory responses. Activated macrophages secrete a number of different inflammatory mediators, including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), prostaglandin E₂ (PGE₂), nitric oxide (NO), and tumor necrosis factor- α (TNF- α) [4-6]. Cytokines, such as IL-1 β ,

IL-6, IL-10, and TNF- α , are soluble proteins that are secreted by the cells of the immune system and can alter the properties of different cell types and provide essential communication signals for motile cells of the immune system [7]; excessive and uncontrolled production of these inflammatory cytokines may lead to serious systemic complications, such as microcirculatory dysfunction, tissue damage and septic shock, which can exact a high mortality [8].

Prostaglandins (PG) are generated by a variety of cell types, including activated macrophages [9]. The rate-limiting enzyme in PG synthesis is cyclooxygenase (COX). COX-2 is induced by several stimuli, including growth factors, mitogens, cytokines, and tumor promoters. Its uncontrolled activity has been theorized to perform an important function in the pathogenesis of a host of chronic inflammatory disorders [9,10]. NO is synthesized from L-arginine by nitric oxide synthase (NOS). Among three distinct NOS isoforms, iNOS is known to be the most important with regard to the regulation of inflammatory responses [11-13]. These inflammatory mediators are critically involved in the pathogenesis of a variety of human inflammatory diseases, including inflammatory bowel dis-

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Corresponding to: Wonyong Kim, Department of Microbiology, College of Medicine, Chung-Ang University, 84, Heukseok-ro, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-820-5685, (Fax) 82-2-822-5685, (E-mail) kimwy@cau.ac.kr



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ABBREVIATIONS: NO, nitric oxide; IL, interleukin; PGE₂, prostaglandin E₂; LPS, lipopolysaccharide; iNOS, inducible NO synthase; COX, cyclooxygenase; MRSA, methicillin-resistant *Staphylococcus aureus*; CRAB, carbapenem-resistant *Acinetobacter baumannii*; TNF- α , tumor necrosis factor- α ; PBR, Polyphenol of black raspberry root; PBF, Polyphenol of black raspberry fruit.

ease, multiple sclerosis and rheumatoid arthritis.

Currently, the emergence of multiple-antimicrobial-resistant bacteria, such as *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* (CRAB), and biological agents (namely *Bacillus anthracis*), represent a serious worldwide health problem [14]. Of these organisms, MRSA has become particularly troublesome in hospitals and the community [15]. High incidences of CRAB infections and hospital-wide outbreaks have been reported from many countries in recent years [16,17]. *B. anthracis*, the most notorious biological agent often referred to as “anthrax,” has been widely used in weapons that could cause disease and death in sufficient numbers to cripple a city or region [18].

Herbal medical approaches against a variety of diseases utilize a number of fruits and vegetables, and the use of these materials in medicine remains a vital field of activity in Oriental countries. Previously, an anti-inflammatory response from the fruit polyphenols of the black raspberry was reported in murine macrophage cells [19]. However, the anti-inflammatory and antimicrobial activities of the root polyphenols of this plant have not yet been evaluated. Recently, plant polyphenols were reevaluated by examining the root polyphenols, which were reported to have many properties, including anti-inflammatory and antidote effects [20]. Therefore, in this study, the biological activities of the polyphenols of black raspberry root were evaluated and compared with those of the fruit polyphenols. We examined the effects of black raspberry polyphenols from two distinct plant parts on the production of IL-1 β , IL-6, IL-10, TNF- α , NO, PGE₂, iNOS, and COX-2 in RAW 264.7 cells stimulated by lipopolysaccharide (LPS) and showing anti-bacterial effects against multiple-antimicrobial-resistant bacteria.

METHODS

Plant material and extraction

Black raspberry root and unripe fruits were purchased from the Yakryeong (Drug) Market (Seoul, Korea). Dried plant materials (100 g) were extracted with 80% ethanol under reflux for 24 hours, and the supernatants were filtered using Whatman filter paper (Macherey-Nagel, Germany). The filtered extracts were evaporated with a rotary evaporator (Eyela, Japan) under reduced pressure, yielding a viscous polyphenol solution. The concentrated polyphenols were freeze-dried.

Cell viability assay

RAW 264.7 murine macrophages were acquired from the Korea Cell Bank (Seoul, Korea) and cultured in DMEM (Lonza, Walkersville, MD, USA) containing 10% heat-inactivated FBS (GIBCO BRL, Grand Island, NY, USA) and 20 μ g/ml of gentamycin (GIBCO BRL) at 37°C in a water-jacketed CO₂ incubator (Model 3111, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was determined with 0, 1, 5, 10, 25, 50, and 100 μ g/ml samples of polyphenols of the root and fruits. RAW 264.7 cells at 5×10^5 cells/well were seeded in 24-well plates. After 24 hr, the cells were treated with serial concentrations of test compounds in 1 ml of serum-free media. After 24-hr treatments, the cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Media were replaced by 500 μ l of fresh serum-free media containing 0.5 mg/ml of MTT (Sigma-Aldrich, St. Louis, MO, USA). After 1 hr of incubation at 37°C with a 5% CO₂ incubator, MTT-containing media were removed, and the reduced formazan dye was solubilized by adding 500 μ l of dimethyl sulfoxide (DMSO) to each well. After gentle mixing, the absorbance was monitored at 590 nm using an Infinite[®] F200 plate reader (Tecan, Männedorf, Switzerland).

Nitrite determination

RAW 264.7 cells were plated at a density of 5×10^5 cells in 24-well cell culture plates with 1 ml of culture medium and then incubated for 24 hr. The cells were treated with 0, 1, 5, 10, 25, 50, and 100 μ g/ml of various polyphenols in LPS (0.1 μ g/ml) and incubated for an additional 24 hr. The quantity of nitrite generated was measured using the Griess reagent system (Sigma-Aldrich, St. Louis, MO, USA). Equal volumes of culture supernatant and Griess reagent were mixed and incubated for 10 min at room temperature. The absorbance was measured at 540 nm on a spectrophotometer and compared to a nitrite standard curve to determine the nitrite concentration in the supernatants.

Measurement of IL-1 β , IL-6, IL-10, PGE₂, and TNF- α

The levels of IL-6, IL-1 β , PGE₂, and TNF- α in the supernatants from macrophage cultures were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). RAW 264.7 cells were incubated with LPS (0.1 μ g/ml) in the presence of different concentrations of polyphenols for 24 hr. The supernatants were collected and stored at -80°C before analysis.

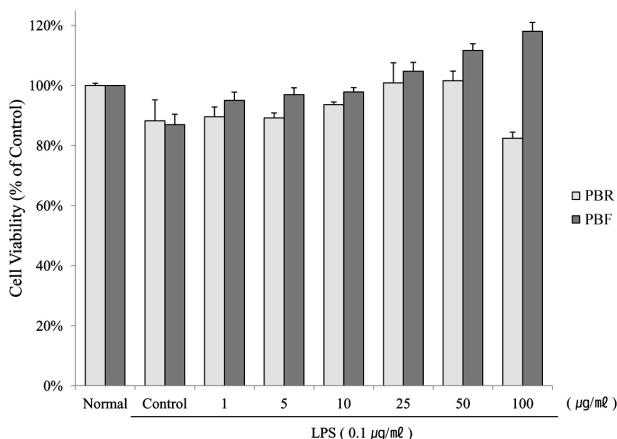


Fig. 1. The cell viability of Raw 264.7 cells with the polyphenol of black raspberry at different concentrations. Cells were treated for 24 hr with 0, 1, 5, 10, 25, 50 or 100 μ g/ml of polyphenols in the presence of 0.1 μ g/ml LPS. The normal group was treated with media only. The results are expressed as the mean \pm SE from three independent experiments. PBR, polyphenol of black raspberry root; PBF, polyphenol of black raspberry fruit.

Evaluation of iNOS, COX-2 using real-time PCR

RAW 264.7 cells were incubated with LPS (0.1 $\mu\text{g/ml}$) in the presence of different concentrations of the polyphenols for 24 hr. Total RNA was extracted from cultured cells using the Trizol method (Gibco BRL). Reverse transcription of the RNA was performed using AMV reverse transcriptase (PROMEGA, Madison, WI, USA). Real-time polymerase chain reaction (PCR) assay was performed and monitored using SYBR Green chemistry with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate to confirm reproducibility. The amount of mRNA in each sample was normalized using that of the mean β -actin levels. Primer sequences were as follows: iNOS, forward primer 5'-TCCTACACCACACCAAAC-3', reverse primer 5'-CTCC-AATCTCTGCCTATCC-3'; COX-2, forward primer 5'-CCTC-TGCCGATGCTCTTCC-3', reverse primer 5'-TCACACTTAT-ACTGGTCAAATCC-3' [21]; β -actin, forward primer 5'-TG-AGAGGGAAATCGTGCGTGAC-3', reverse primer 5'-GCT-CGTTGCCAATAGTGATGACC-3' [22].

In vitro antimicrobial activity assay

Antimicrobial activity against ten microorganisms was primarily determined by the agar disc diffusion method using the diameter of the clear zone. MRSA CAU 11001-CAU 11005 and CRAB CAU 10210-CAU 10204 were isolated in our laboratory from patients. *B. anthracis* ATCC 14578^T was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The strains were grown aerobically on Mueller Hinton agar (Difco, Detroit, MI, USA) at 37°C for 18 hr. Cultured-media from test microorganisms were put in sterilized paper discs (10 mm) and placed on the inoculated agar surface. The cultured media from the isolated bacteria were used to fill in the paper discs (100 $\mu\text{l/disc}$). After pre-diffusion at room temperature for 1 hr, the plates were incubated at 37°C for 19 hr. The non-cultured-media filled in the paper discs were used as controls.

Determination of the minimum inhibitory concentrations (MICs)

A broth microdilution susceptibility assay was conducted in accordance with NCCLS (2009) guidelines for the minimal inhibitory concentration (MIC) determinations. All tests were performed in Mueller Hinton agar (Difco), and the strains were cultured overnight at 37°C. Bacterial suspensions were diluted to match the 0.5 MacFarland standards (approximately 1.5×10^8 CFU/ml). The extracted compounds were dissolved in DMSO, and two-fold serial dilutions were prepared then added to the BHI media (final concentration range: 15.6~1,000 $\mu\text{g/ml}$). The culture media were incubated under normal atmospheric conditions for 24 hr at 37°C. Bacterial growth was indicated by the presence of a white pellet on the bottom of the tube. The MIC value was defined as the lowest concentration of polyphenols at which no visible growth could be observed.

RESULTS

Cell viability MTT

The cytotoxic effects of the root and unripe fruit polyphenols of black raspberry were evaluated in RAW 264.7 cells using MTT assays. Polyphenols were measured at treatment concentrations in the cell culture system. As shown in Fig. 1, the cytotoxicity of the polyphenols (up to a concentration of 100 $\mu\text{g/ml}$) was not obvious after a 24-hr incubation.

Generation of NO and PGE₂ in LPS-stimulated RAW 264.7 cells

The inhibitory effects of the root and unripe fruit polyphenols of black raspberry were evaluated on the production of nitrite, a stable metabolite of NO, and PGE₂ in RAW 264.7 cells that had been challenged with LPS in the presence or absence of polyphenols. As shown in Fig. 2, both

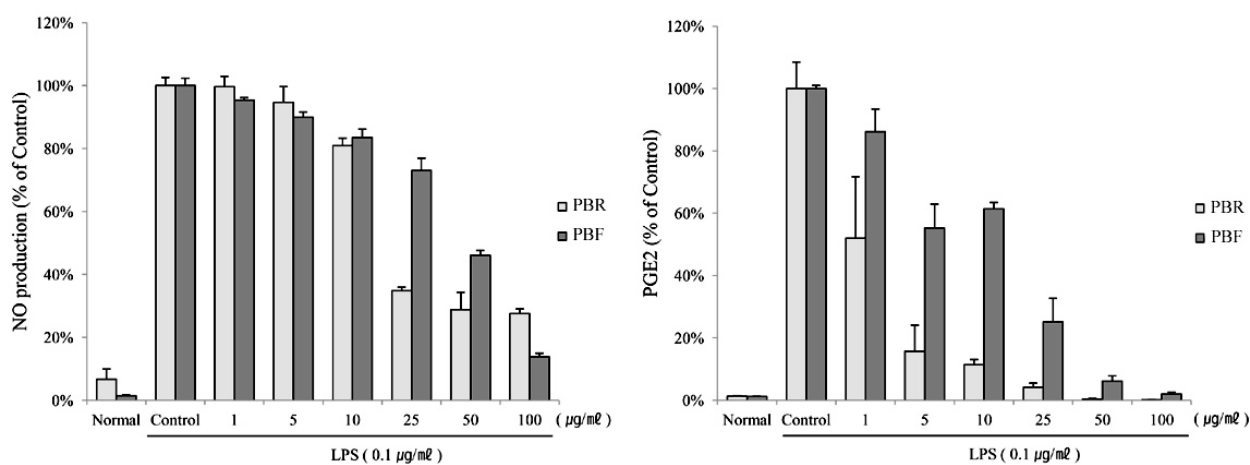


Fig. 2. Suppression of LPS-induced NO and PGE₂ production in RAW264.7 cells by the polyphenol of black raspberry. Cells were treated for 18 hr with 0, 1, 5, 10, 25, 50 or 100 $\mu\text{g/ml}$ of polyphenols in the presence of 0.1 $\mu\text{g/ml}$ LPS. The normal group was treated with media only. The results are expressed as the mean \pm SE from three independent experiments. PBR, polyphenol of black raspberry root; PBF, polyphenol of black raspberry fruit.

polyphenols effectively suppressed LPS-induced nitrite production in a dose-dependent manner. Consistent with the observations with regard to nitrite accumulation, the root polyphenol profoundly inhibited PGE₂ production. NO production in the treatment of the root polyphenol was significantly lower than that of the fruit polyphenols at 25 and 50 $\mu\text{g/ml}$ concentrations. The NO in LPS-stimulated RAW 264.7 cells was exposed to 25 $\mu\text{g/ml}$ of the root polyphenols, and the fruit polyphenols were measured at 34.9 ± 1 and $73 \pm 3.9\%$ (% of the control), respectively. PGE₂ production with the treatment of the root polyphenol was lower in LPS-stimulated RAW 264.7 cells than the fruit polyphenol at the various concentrations (1, 5, 10, 25, 50, and 100 $\mu\text{g/ml}$). PGE₂ production exposed to 5 $\mu\text{g/ml}$ of the fruit polyphenols and the fruit polyphenol measured at 15.7 ± 8.4 and $55.2 \pm 7.8\%$ (% of the control), respectively. These results show that the polyphenols of the black raspberry exerted an anti-inflammatory effect by ameliorating the production of inflammatory mediators, including NO and PGE₂. In particular, PGE₂ was dramatically reduced (47%) compared to NO and other inflammation factors in the root polyphenols treated with 1 $\mu\text{g/ml}$ on the RAW 264.7 cells.

Effect of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells

The root and unripe fruit polyphenols of the black raspberry were evaluated and compared with regard to the production of IL-1 β , IL-6, IL-10, and TNF- α (major pro-inflammatory cytokines) in LPS-stimulated RAW 264.7 cells. As shown in Fig. 3, IL-1 β , IL-6 and IL-10 production was diminished dramatically following treatment with both polyphenols. In particular, root polyphenols revealed 10 $\mu\text{g/ml}$ concentrations, which was lower than fruit polyphenols with regard to IL-1 β , IL-6 and IL-10 production. The percentage of inhibition in the exposure of 10 $\mu\text{g/ml}$ of root and fruit polyphenols were as follows: 47.2 ± 5.8 and $62.5 \pm 5.1\%$ (% of the control), respectively, for IL-1 β ; 29.4 ± 3.4 and $62.3 \pm 4.5\%$ (% of the control), respectively, for IL-6; and 49.6 ± 13.4 and $87.1 \pm 6.9\%$ (% of the control), respectively, for IL-10. The inhibitory effects of the 25 $\mu\text{g/ml}$ of root polyphenols on IL-1 β production were similar to the effects on IL-6 in that 25 $\mu\text{g/ml}$ of the root polyphenols inhibited IL-10 production by 75~80%. However, no inhibition of

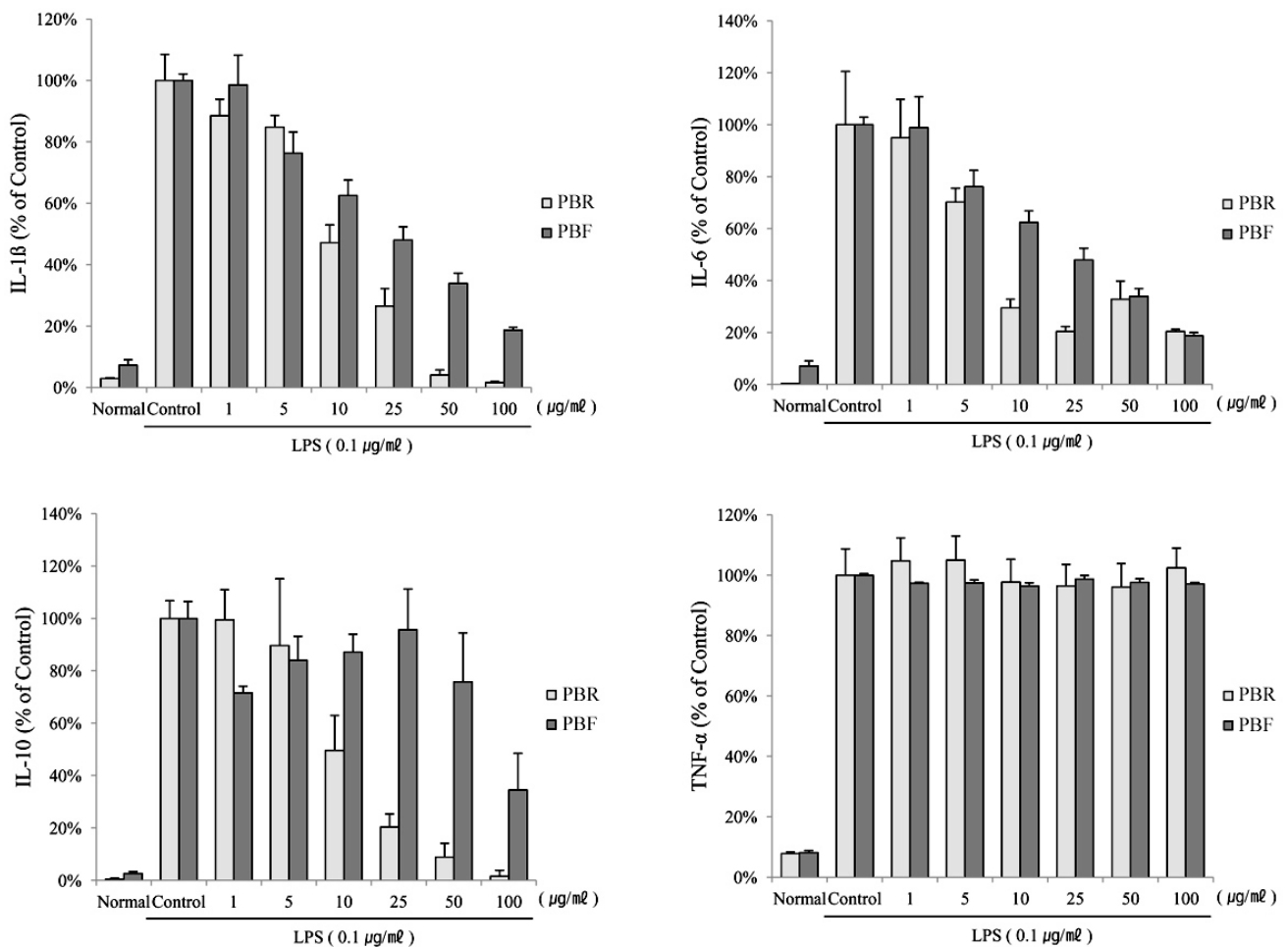


Fig. 3. Suppression of LPS-induced cytokine production (i.e., IL-1 β , IL-6, IL-10, TNF- α) in RAW264.7 cells by the polyphenol of black raspberry. RAW264.7 cells were treated for 18 h with 0, 1, 5, 10, 25, 50 or 100 $\mu\text{g/ml}$ of polyphenols in the presence of 0.1 $\mu\text{g/ml}$ LPS. The normal group was treated with media only. The cell culture media were then collected. The results are expressed as the mean \pm SE from three independent experiments. PBR, polyphenol of black raspberry root; PBF, polyphenol of black raspberry fruit.

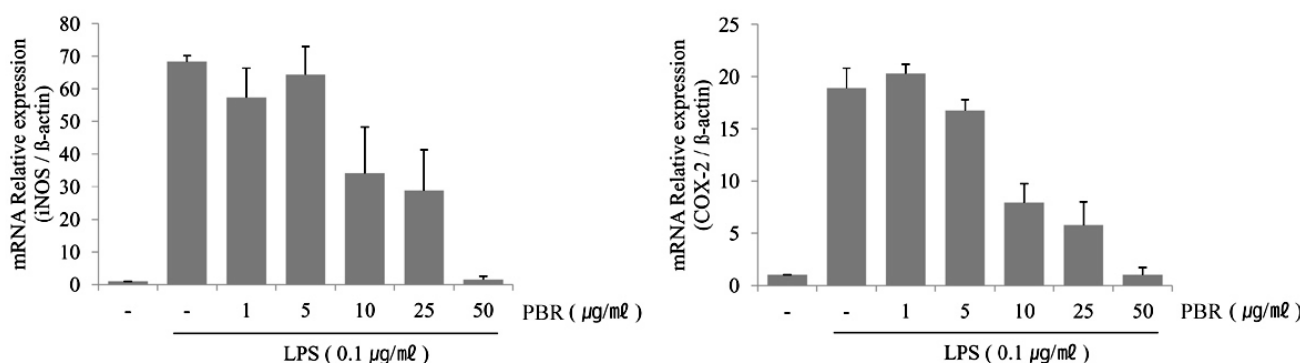


Fig. 4. Effects of PBR on mRNA levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The cells were treated with LPS or LPS plus black raspberry root polyphenol at concentrations indicated in Fig. 4. After 24 hr of incubation, total RNA was subjected to qRT-PCR. The RT products were labeled with SYBR Green dye. Relative iNOS and COX-2 mRNA expression ($2^{-\Delta\Delta Ct}$) were determined by real-time PCR and calculated by the Ct value for iNOS and COX-2 from β -actin mRNA. $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}) - (Ct_{\text{control}} - Ct_{\beta\text{-actin}})$. Each value represents the mean \pm SE of three independent experiments.

Table 1. Antibacterial test results of PBR^{a)} in disc diffusion assay

Microorganism	Strain	Polyphenol concentration (mg/disc)					
		15	7.5	3.75	1.875	0.9375	0.46875
<i>Staphylococcus aureus</i> (MRSA)	CAU 11001	16 ^{b)}	14	12	ND ^{c)}	ND	ND
	CAU 11002	20	18	17	12	ND	ND
	CAU 11003	22	20	17	13	ND	ND
	CAU 11004	20	17	14	11	ND	ND
	CAU 11005	16	17	14	ND	ND	ND
Carbapenem-resistant <i>Acinetobacter baumannii</i> (CRAB)	CAU 10201	16	14	12	ND	ND	ND
	CAU 10202	16	15	11	ND	ND	ND
	CAU 10203	15	14	11	ND	ND	ND
	CAU 10204	16	12	12	ND	ND	ND
<i>Bacillus anthracis</i>	ATCC 14578 ^T	18	16	14	13	11	ND

^{a)}PBR, polyphenol of black raspberry root; ^{b)}Inhibition zones including the diameter of the paper disc (10 mm); ^{c)}ND, not detected.

TNF- α was observed in either polyphenol.

Quantitative RT-PCR of iNOS and COX-2

The mRNA levels of iNOS and COX-2 were determined using qRT-PCR in order to confirm the inhibitory effects against NO and PGE₂ production in LPS-stimulated RAW 264.7 cells treated with root polyphenols. As shown in Fig. 4, iNOS and COX-2 expressions were reduced, which is similar to the results shown in Fig 2. The findings demonstrated that 10, 25 and 50 μ g/ml of polyphenols significantly decreased iNOS mRNA levels by 51, 59 and 99%, respectively, as compared to the LPS-treated positive control cells. The effects of COX-2 revealed that 10, 25 and 50 μ g/ml of polyphenols resulted in significant decreases in mRNA levels of COX-2 by 61, 73 and 100%, respectively, as compared to the LPS-treated positive control cells.

Antimicrobial activity

As shown in Table 1, the root polyphenols were effective at inhibiting all strains tested, especially MRSA and *B. anthracis*. The disc diffusion values of the 15 mg/ml the root polyphenol were included for the ten strains of MRSA and

CRAB. As shown in Table 1, the diameters for the different strains were 18, 16, 20, 22, 20, 16, 16, 16, 16, 20, 17, and 12 mm after an 18-hr exposure. The MICs of the polyphenols using each strain are shown in Table 2. CRAB CAU 10201, CAU 10202, CAU 10203, and CAU 10204 had MICs of 1,000 μ g/ml, while MRSA CAU 11001, MRSA CAU 11002 and MRSA CAU 11003 had MICs of 250 μ g/ml. *B. anthracis*, MRSA CAU 11004 and CRAB CAU 10205 had MICs of 125 μ g/ml, and MRSA CAU 11005 had a MIC of 62.5 μ g/ml. However, no inhibitory effects were observed in any test strains treated with the fruit polyphenols.

DISCUSSION

Black raspberry fruit has traditionally been used in Oriental medicine, and recent *in vitro* studies have demonstrated its antioxidant, anti-tumorigenic and anti-inflammatory effects [23-26]. The roots of many plants have biological activity with anti-inflammatory, antioxidant, antibacterial, anti-fungal, and antidote effects [20]; therefore, the black raspberry may be useful for the food and cosmetic industries and/or as pharmaceuticals. The increased consumption of the root of black raspberry has prompted farm-

Table 2. Minimum inhibitory concentrations of PBR against pathogenic bacteria

Microorganism	Strain	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i> (MRSA)	CAU 11001	250
	CAU 11002	250
	CAU 11003	≤ 250
	CAU 11004	125
	CAU 11005	62.5
Carbapenem-resistant <i>Acinetobacter baumannii</i> (CRAB)	CAU 10201	$> 1,000$
	CAU 10202	1,000
	CAU 10203	$> 1,000$
	CAU 10204	1,000
<i>Bacillus anthracis</i>	ATCC 14578 ^T	125

ers to expand the harvest area of this plant, and the growing area in the Jeonbuk province in South Korea has increased year by year. The root of this plant wastes after fruit harvest, so it can be gathered more easily than other plant root materials. However, its biological activities have not been studied until recently.

The present study examined the bioactive natural constituents exerting anti-nociceptive and anti-inflammatory activities from the polyphenols of the black raspberry by activity-guided fractionation using ethyl acetate and butyl alcohol. These fractions showed significant anti-nociceptive effects as assessed by writhing-, hot plate- and tail flick tests in mice and rats, as well as an anti-inflammatory effect in rats with carrageenan-induced edema [27]. Recently, these anti-inflammatory effects were examined by comparison of polyphenols of the unripe, semi-ripened and ripe fruits of this plant. Unripe and semi-ripened fruits were found to reduce the production of NO and PGE₂ as well as pro-inflammatory cytokines. However, ripe fruit exerted no inhibitory effects against the production of NO and IL-6 [19].

Drugs that suppress or inhibit the expression of these inflammation-associated genes can be considered to harbor therapeutic potential for the prevention of inflammatory reactions and diseases. In this study, it was demonstrated that root polyphenols down-regulated NO, PGE₂, IL-1 β , IL-6, and IL-10 production in LPS-stimulated RAW264.7 cells to a greater extent than fruit polyphenols. These findings indicate that the roots of this plant may prove to have a useful therapeutic approach in a variety of inflammatory diseases.

In addition, as a result of anti-superbacterial activity, root polyphenols were more effective than fruit polyphenols against MRSA, CRAB and *B. anthracis*. Notably, CRAB was inhibited by root polyphenols. Carbapenems have been reported as the most appropriate choice for the treatment of infections by multidrug-resistant *A. baumannii*. The most clinically significant carbapenemases are metalloenzymes, and resistance to carbapenems in *A. baumannii* is also conferred by some carbapenemases (Ambler class D) that weakly hydrolyze imipenem and meropenem [28]. However, resistance to these molecules has increased greatly worldwide [29,30]. The present findings clearly indicate that root polyphenols are a candidate for powerful antimicrobial biocides for the control of multi-drug resistance or harmful bacteria. This is the first report of this potential application.

In conclusion, the results obtained from this study can be expected to serve as the basis for understanding the

pharmacological effects of black raspberry and could be used to develop new pharmaceutical materials.

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