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Polyphenol Compounds and Anti-inflammatory Activities of Korean Black Raspberry (*Rubus coreanus* Miquel) Wines Produced from Juice Supplemented with Pulp and Seed

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ABSTRACT: Three types of Korean black raspberry wine were produced via alcoholic fermentation from juice, juice−pulp, and juice−pulp−seed, respectively. These wines were compared in terms of their anti-inflammatory activities and polyphenol contents. The total content of polyphenol compounds in wines was increased by 22.4% after supplementation with pulp and by 56.7% after supplementation with both pulp and seed. The reduction rate of NO evolution was highest in the order juice−pulp−seed wine, juice−pulp wine, and juice wine. Addition of the juice−pulp−seed wine at a level of 62.5−500 mg/L decreased the NO evolution rate by 40.5−94.2%. Eight fractions were obtained from juice−pulp−seed wine via ethyl acetate extraction and silica gel chromatography. Of these, the AF fraction, which exhibited the highest in vitro anti-inflammatory activity, exerted inhibitory effects on ear edema, writhing response, and vein membrane vascular permeability in mice. 3,4-Dihydroxybenzoic acid accounted for 37.6% of the total polyphenol content in the AF fraction.

KEYWORDS: *Korean black raspberry wine, Rubus coreanus Miquel, polyphenols, anti-inflammatory, 3,4-dihydroxybenzoic acid*

INTRODUCTION

Korean black raspberry (*Rubus coreanus* Miquel) belongs to the Rosaceae family and is cultivated in Southeast Asia.[1] Juices and wines made from the fruits have been popular as traditional beverages in Korea. The fruits contain flavonoids, tannins, phenolic acids, organic acids, triterpenoids, and polyphenols including lots of volatile compounds.[2−4] There are reports[5,6] that the major phenolic compounds of black raspberry fruits are protocatechuic acid, epicatechin, ellagic acid, and rutin, and their minor compounds are catechin, quercetin, ferulic acid, etc. Supplementation with pulp and/or seeds of black raspberry significantly increases the phenolic compound content and quality of these wines.[4]

Raspberry fruits have been used for the treatment of impotence, spermatorrhea, enuresis, asthma, and skin disease.[7] There is a report[8] that raspberry fruits have anti-inflammation, antifatigue, antistomach ache, antirheumatism, and antioxidant activities. The fruit inhibits nitric oxide (NO) formation and suppresses formation of activated oxygen molecules, resulting in an anticancer effect.[9,10] NO, a reactive and unstable free radical, is a mediator of inflammation that is produced from arginine by nitric oxide synthase (NOS).[11] Inducible NOS (iNOS) is one of the three isoforms of NOS and increases the NO evolution rate when exposed to lipopolysaccharide (LPS). Excessive formation of NO causes both an inflammatory reaction and tissue damage.

For the treatment of inflammatory diseases, compounds suppressing excessive NO formation have been sought from a variety of sources. N^G-Monomethyl-L-arginine (L-NMMA), aminogluanidine, and dexamethansone are known inhibitors of iNOS.[12,13] Until now, there have been few reports concerning the anti-inflammatory effects of Korean black raspberry wines in mice. In this study, three types of Korean black raspberry wine were produced via fermentation from different media, including fruit, fruit and pulp, and juice and pulp and seed. The polyphenol contents and the anti-inflammatory activities of the resultant wines and their purified fractions were analyzed and compared. The inhibition rates of NO formation in cells and specifically for ear edema, writhing reaction, and blood diffusion in mice were measured.

MATERIALS AND METHODS

Chemicals and Materials. High-fructose corn syrup for sugar enrichment was purchased from Daesang Inc. (Gunsan, South Korea). Sodium sulfite, which was used for the prevention of oxidation and contamination during the fermentation process, was a product of Shinyo Pure Chemicals Co., Ltd. (Osaka, Japan). Dulbeco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin−streptomycin (P/S), and Hank's balanced salt solution (HBSS) were purchased from Gibco (New York, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), sodium nitrite, LPS, Griess reagent, L-NMMA, dimethyl sulfoxide (DMSO), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), indomethacin, Evans blue, gallic acid, catechin, epicatechin, *p*-coumaric acid, quercetin, chlorogenic acid, ethyl gallate, 4-hydroxybenzoic acid, and syringic acid were obtained from Sigma (St. Louis, MO, USA). Rutin was a product of Wako (Osaka, Japan), and caffeic acid, myricetin, ellagic acid, ferulic acid, kaempferol, and 3,4-dihydroxybenzoic acid (also called protocatechuic acid) were products of Fluka (Buchs SG, Switzerland). Malvidin-3-glucoside was purchased from Indofine

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(Hillsborough, NJ, USA). All other chemicals and solvents were of either first class or high grade.

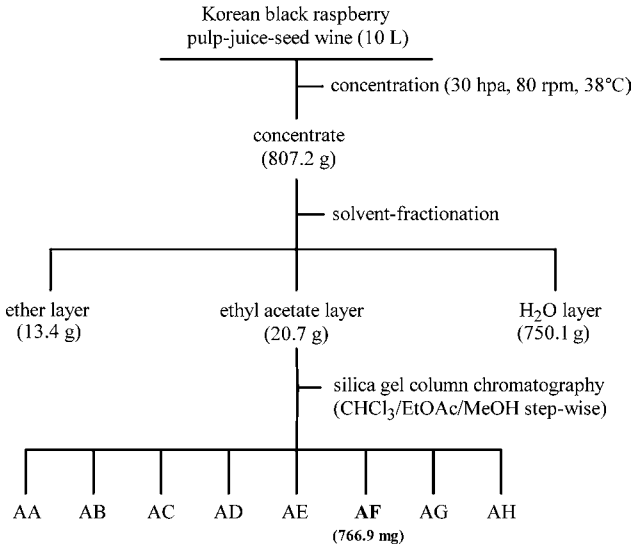
Cell Lines and Mice. The dry yeast of Fermiblanc Arom (*Saccharomyces cerevisiae*; Gist-brocades, Seclin, France) was used for ethanol fermentation. The cell line RAW 264.7-L1, used for measurement of the NO evolution rate, was obtained from the Korean Cell Line Bank (Seoul, Korea).

Female ICR mice (28−32 g) and female BALB/c (19−22 g) were purchased from Daehan Biolink Co. (Eumseoung-gun, Korea). Mice were maintained in a room at 23 ± 2 °C with a relative humidity of 50 ± 10%. Three mice were put in a cage, and solid foods and water were freely available for an adaptation period of 1 week. All mice were handled following the *Guide for the Care and Use of Laboratory Animals*.[14]

Preparation of Black Raspberry Media and Ethanol Fermentation. Korean black raspberry (Bokbunja) fruits were harvested in Gochang province in South Korea. The fruits were divided into 20 kg units, then placed into polypropylene vessels, and quickly cooled to −20 °C. The vessels were kept in a freezer (−20 °C, chest model, Ilshin Biobase, Korea). After frozen black raspberry fruits were thawed in an incubator (KCL1000, Eyela, Japan) at 25 °C, they were mixed by hand. Three kinds of juice solution (juice, juice−pulp, and juice−pulp−seed) were prepared via different filtration procedures. The juice solution was prepared by removing pulp and seed using a 100 mesh sieve. The juice−pulp solution was passed through a 25 mesh sieve to remove the seeds. The juice−pulp−seed solution was not subjected to filtration. The sugar content of all three solutions was approximately 10.2 °Brix.

For fermentation, after 20 L round glass jars (30 × 40 cm) were filled with 12 kg of each solution (juice, juice−pulp, and juice−pulp−seed), the sugar content was adjusted to 20 °Brix by supplementation with high-fructose corn syrup. The media sugar contents were 212.6 g (97.0 g of glucose and 115.6 g of fructose) per liter for juice, 208.6 g (95.4 g of glucose and 113.2 g of fructose) per liter for juice−pulp, and 197.1 g (90.1 g of glucose and 107.0 g of fructose) per liter for juice−pulp−seed. An amount of 70 ppm sodium sulfite was added to the glass jars prior to incubation for 3−4 h. Then, 200 ppm dry yeast was added. Fermentation was performed for 15 days in an incubating room with stirring two times every day. The temperature inside the jars was kept at 25 °C. After fermentation, culture broths were passed through filter paper (NA-050, Advantec). The solutions were placed into 2 L bottles and sealed using a wood stopper. The bottles were kept for 3 months in a storage incubator at 15 °C.

Scheme 1. Procedure for Fractionation of Korean Black Raspberry Wine



Fractionation and Purification of Black Raspberry Wine. As shown in Scheme 1, 10 L of juice−pulp−seed wine was concentrated under vacuum (30 hPa) and then adjusted to 2 L using distilled water. Two liters of both ether and ethyl acetate were added to a separating funnel containing the solution. Thus, three layers (ether, ethyl acetate, and water) were harvested. These procedures were repeated three times, and each layer was grouped. The ethyl acetate layer was further purified using silica gel column chromatography (glass open column, Duran, Germany) in which mixtures of $CHCl_3$/EtOAc/MeOH (10:0:0, 8:2:0, 6:4:0, 4:6:0, 2:8:0, 0:10:0, 0:8:2, 0:6:4, 0:4:6, 0:2:8, and 0:0:10, v/v/v) were eluted as a mobile phase in a stepwise manner. After each fraction (AA−AH) was subjected to TLC, similar fractions were grouped together.

The AF fraction was subjected to HPLC using a μBondapak C_{18} column (10 μm, 125 Å, 7.8 × 300 mm, preparative column, part WAT 084176, Waters, Ireland). The solvent was eluted at a flow rate of 2.0 mL/min under gradient conditions (30 min at 27−70% methanol, 5 min at 70−100% methanol) and then maintained for 15 min. The active AF fraction was further purified (AF3) via HPLC using a Prodigy 5 ODS column (4.6 × 250 mm, 5 μm, Phenomenex) under isocratic conditions by eluting 10% methanol at a flow rate 1.0 mL/min (Scheme 1) with a resultant compound.

Procedure for Analysis of Polyphenol Compounds. Fractions AA−AH (Scheme 1) were qualitatively and quantitatively analyzed for 17 polyphenol compounds following the method of Cantos et al.[15] The standard compounds were gallic acid, catechin, caffeic acid, epicatechin, *p*-coumaric acid, malvidin 3-glucoside, rutin, myricetin, quercetin, 3,4-dihydroxybenzoic acid, ellagic acid, ferulic acid, chlorogenic acid, kaempferol, ethyl gallate, 4-hydroxybenzoic acid, and syringic acid. The polyphenol amounts in Korean black raspberry fruit juice and wines were determined using a UPLC equipped with a PDA detector and AcQuity UPLC BEH C_{18} (2.1 × 100 mm, 1.7 μm particle size) (Waters, Ireland). Five percent formic acid (solvent A) and methanol (solvent B) were eluted at a rate of 0.3 mL/min. The formic acid (%) and methanol (%) gradient was 98:2 at 0 min to 5:95 at 20 min to 98:2 at 25 min.

Study Design of Samples. The activities of various wine samples were tested using a cell line or mice. In tests using RAW 264.7 cells, three types of samples were used: (1) original wines, that is, juice wine, juice−pulp wine, and juice−pulp−seed wine; (2) fractions from ether and ethyl acetate extractions of juice−pulp−seed wine; (3) fractions from ethyl acetate extraction and silica gel chromatography of juice−pulp−seed wine. In tests using mice, the AF fraction from ethyl acetate extraction and silica gel chromatography of juice−pulp−seed wine was used for determination of different inhibitory effects on the acetic acid-induced writhing response, vascular permeability, and TPA-induced ear edema.

Measurement of the NO Evolution Rate. The Griess method was used.[7] RAW-264.7 cells were cultivated for 48 h in T-flasks containing 10 mL of DMEM supplemented with 10% FBS and 1% P/S in a 5% CO_2 incubator (Sanyo, Japan) at 37 °C. Then, they were collected using a scraper and centrifuged for 5 min at 1000 rpm (Hettich, PM 6100, Germany). Cells were added at 2×10^5 per well to 96-well plates and then incubated for 2 h. Amounts of 0−500 μg/mL of wine sample and 1 μg/mL of LPS per milliliter were added to the plates. After the plates were cultivated for 24 h, 100 μL of cell culture broth was mixed with 100 μL of Griess reagent. The amounts of evolved NO were determined via an absorbance measurement at 550 nm using a microplate reader (Bio-Rad, USA). The wine samples tested were specified in the previous section, and 10 μM L-NMMA was used as a control.

Determination of the Inhibitory Effect on Ear Edema in Mice. The methods of Chem et al.[16] and Moronkola et al.[17] were modified to determine the inhibitory effect of wine on ear edema in mice after acute inflammation in 5-week-old female BALB/c mice was induced via TPA. The ear thickness before and after inflammation was measured using a digital micrometer (Mitutoyo, Japan), and differences were expressed as the ear swelling response (ESR). After TPA was dissolved at 2 μg/20 μL in acetone, 10 μL of the solution was applied to the inside and outside of the mouse ear. Thirty minutes

later, a sample of each wine at concentrations of 10 μg/20 μL (AF 10, n = 12) and 20 μg/20 μL (AF 20, n = 12) was applied to the same mouse ear. A positive control group (n = 12) was treated only with TPA, and a normal control group (n = 12) was treated with acetone. Seven hours after the TPA treatment, the ear thickness was measured, and the value was converted into an inhibitory rate as

$$\text{inhibitory rate of ear edema (\%)} = \left[1 - \left(\frac{T_{sample} - T_{blank}}{T_{control} - T_{blank}}\right)\right] \times 100 \quad (2)$$

where T_{blank} is the difference in ear thickness of the mouse group without any treatment, $T_{control}$ is the difference for the group treated with TPA, and T_{sample} is the difference for the group treated with both TPA and wine.

Inhibition Effect of Wine on the Writhing Response. The method of Koster et al.[18] was used to determine the inhibitory effect of wine on writhing response in mice. Five-week-old female ICR mice were divided into the four groups of (1) a control (treated with 0.9% saline solution per kilogram, n = 8), (2) AF-50 (treated with 50 mg of AF per kilogram, n = 8), (3) AF-100 (treated with 100 mg of AF per kilogram, n = 8), and (4) INDO (indomethacin as an authentic inhibitor; treated with 20 mg per kilogram, n = 8). After a 20 h fast, mice were weighed and fed orally with samples including AF. After 1 h, 0.7% acetic acid was injected abdominally at 100 μL/10 g for all groups. After 5 min, the struggle reaction was evaluated for 15 min by two observers for each mouse.

Inhibition Effect of Wine on Vein Permeability. The inhibitory effect of wine on protrusion of capillary vessels in mice was evaluated using the modified Khanna method.[19] Five-week-old female ICR mice were treated in the same manner described in the previous section. Thirty minutes after oral intake of wine, 0.7% acetic acid in a saline solution was injected into the abdomen at 100 μL/10 g for all groups to enhance the permeability of veins. Thirty minutes later, 100 μL of 4% Evans blue was injected into the caudal vein. After 30 min, mice were killed by dislocating the cervical vertebrae. After 5 mL of a saline solution was injected into the abdomen and the mouse was shaken, Evans blue eluted from the abdomen was collected after 10 min of centrifugation (Hanil, Korea), and the absorbance was measured at 590 nm using a spectrophotometer. The inhibitory rate of diffusion across capillary veins (%) was calculated as

$$\text{inhibitory rate (\%)} = \left\{1 - \left(\frac{Ab_{sample} - Ab_{blank}}{Ab_{control} - Ab_{blank}}\right)\right\} \times 100 \quad (3)$$

where Ab_{blank} is the absorbance without any treatment, $Ab_{control}$ is the control treated with acetic acid, and Ab_{sample} is the control treated with both acetic acid and wine.

Statistical Analysis. Experiments were repeated three times, and results were expressed as the mean ± standard deviation. ANOVA and Tukey's tests for data comparison were performed with a significance value of $p < 0.05$.

RESULTS

Inhibitory Effect of Korean Raspberry Wines on NO Evolution in Cells. The anti-inflammatory effect of Korean black raspberry wines was evaluated on the basis of a reduction in the NO evolution in RAW 264.7 cells. When LPS was added to cause cellular inflammation, an NO yield of 31.8 μM was obtained (Table 1). However, when L-NMMA, an inhibitor of NO evolution, was added as a supplement to LPS, the NO yield was decreased to 6.2 μM, corresponding to an 86.2% reduction in the rate. The three kinds of Korean raspberry wine were also used as supplements for NO evolution inhibition. When the content of juice−pulp−seed wine increased from 62.5 to 500 μg/mL, the NO yield decreased from 19.8 to 3.8 μM, corresponding to a 40.5−94.2% reduction in the rate. When juice−pulp wine and juice wine were added as supplements at 500 μg/mL, their NO reduction rates were 86.1 and 79.5%, respectively. The anti-inflammatory activity of Korean black raspberry wine was enhanced by supplementation of pulp and seeds.

Table 1. Inhibitory Effect of Korean Black Raspberry Wines on NO Evolution in RAW 264.7 Cells Exposed to LPS

sample[a]	content (μg/mL)	NO evolution yield[b] (μM)	inhibition rate (%)
nontreated		2.09 ± 1.18	0
LPS	1	31.84 ± 8.07	100
L-NMMA	10	6.20 ± 2.40	86.18
juice−pulp−seed wine	500	3.82 ± 1.04a	94.18
	250	7.21 ± 1.87b	82.79
	125	15.53 ± 3.01c	54.82
	62.5	19.80 ± 3.45c	40.47
juice−pulp wine	500	6.24 ± 1.21a	86.05
	250	12.07 ± 2.43b	66.45
	125	23.62 ± 4.07c	27.63
	62.5	27.34 ± 5.18c	15.12
juice wine	500	8.17 ± 1.76a	79.56
	250	11.81 ± 2.01a	67.32
	125	20.16 ± 3.53b	39.26
	62.5	25.72 ± 4.21b	20.57

[a]LPS = lipopolysaccharide; L-NMMA = L-monomethyl-L-arginine. [b]NO evolution yields of RAW 264.7 cells after supplementation of samples were measured using the Griess method. The amounts of evolved NO were determined using an absorbance measurement at 550 nm with a microplate reader. Identical experiments were repeated three times and expressed as the mean ± standard deviation.When letters in each wine are not same, values are significantly different ($p < 0.05$) (Tukey's test).

Analysis of the Polyphenol Compounds of Korean Black Raspberry Wines. The key polyphenol compounds of fruit juice, juice wine, juice−pulp wine, and juice−pulp−seed wine were analyzed (Table 2). The fruit juice contained rutin, epicatechin, catechin, malvidin 3-glucoside, 3,4-dihydroxybenzoic acid, and myricetin in large amounts (20−60 mg/L), and gallic acid, p-coumaric acid, and quercetin in small amounts (5−12 mg/L). During fermentation, the amounts of rutin, 3,4-dihydroxybenzoic acid, caffeic acid, gallic acid, and p-coumaric acid were increased by 27−188%, whereas those of catechin, epicatechin, malvidin-3-glucoside, myricetin, and quercetin were decreased by 5−30%. The 3,4- dihydroxybenzoic acid content was increased approximately 3 times. Supplementation with pulp caused a 3−70% increase in the amount of most polyphenol compounds, whereas seed caused an additional 32−241% increase. The amounts of gallic acid, quercetin, myricetin, and malvidin-3-glucoside were greatly increased, but the rutin and caffeic acid amounts were not significantly changed.

Fractionation of the Juice−Pulp−Seed Wine. Juice−pulp−seed wine, which showed the highest inhibitory effect on NO evolution, was subjected to ether and ethyl acetate extraction, and the two resultant extracts were tested for their inhibitory effects. As shown in Figure 1, the NO evolution rate decreased with an increasing content of both extracts. Because the ethyl acetate extract was more inhibitory than the ether extract (a 97.5 to 60.0% reduction in the rate at 100 μg/mL),

Table 2. Contents of Polyphenol Compounds in Korean Black Raspberry Wines[a]

		wines		
polyphenol (mg/L)	juice	juice	juice−pulp	juice−pulp−seed
3,4-dihydroxybenzoic acid	21.87 ± 0.54a	63.10 ± 0.39b	78.94 ± 0.41c	84.30 ± 0.30d
gallic acid	11.67 ± 0.33a	16.16 ± 0.15b	26.22 ± 0.37c	55.20 ± 0.96d
catechin	48.89 ± 0.23b	46.27 ± 0.63a	57.34 ± 0.42c	65.99 ± 0.34d
caffeic acid	11.81 ± 0.46a	18.10 ± 0.60b	24.43 ± 0.53c	24.09 ± 0.13c
epicatechin	58.89 ± 0.38b	54.77 ± 0.54a	61.15 ± 0.82c	83.42 ± 0.30d
p-coumaric acid	9.70 ± 0.22a	12.34 ± 0.33b	12.82 ± 0.50b	17.31 ± 0.30c
malvidin 3-glucoside	35.87 ± 0.28b	27.44 ± 0.62a	46.84 ± 0.23c	77.33 ± 0.26d
rutin	60.71 ± 0.46a	80.36 ± 0.86c	83.12 ± 0.22d	75.70 ± 0.27b
myricetin	19.42 ± 0.43c	13.66 ± 0.39a	16.15 ± 0.27b	32.55 ± 0.10d
quercetin	4.73 ± 0.12b	3.58 ± 0.26a	3.97 ± 0.29a	10.44 ± 0.09c
total	283.60	335.77	411.05	526.36

[a]Identical experiments were three times repeated and expressed as the mean ± standard deviation. When letters in the same row are not the same, values are significantly different ($p < 0.05$) (Tukey's test).

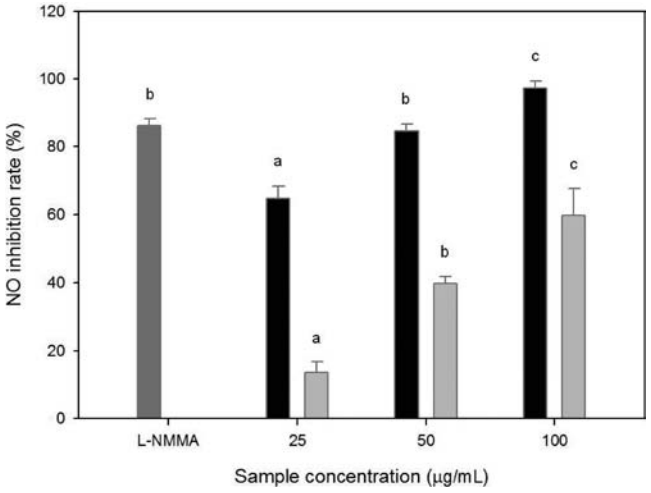


Figure 1. Inhibitory effect of the ether (gray bars) and ethyl acetate (black bars) extracts from juice−pulp−seed wine on NO evolution in RAW 264.7 cells exposed to LPS. After three independent experiments were performed, values were averaged and expressed as the mean ± standard deviation. NO inhibition rates were expressed as a percent relative to the amount of NO evolved after exposure to LPS. When the letters for each extract are not the same, the values are significantly different ($p < 0.05$) (Tukey's test).

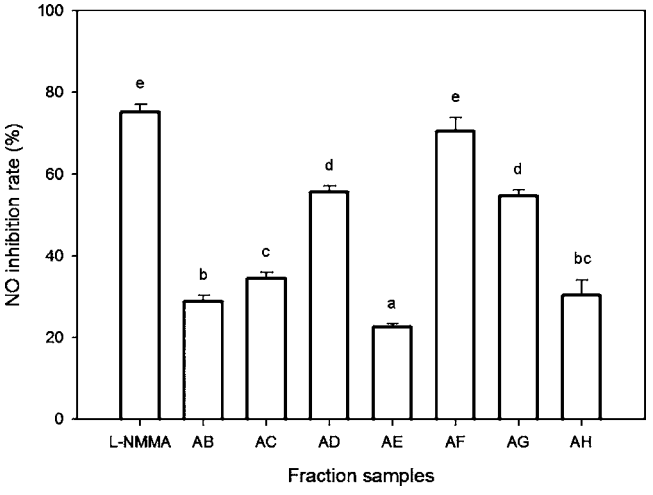


Figure 2. Inhibitory effect of fractions from ethyl acetate extraction and silica gel chromatography on NO evolution in RAW 264.7 cells exposed to LPS. After three independent experiments were performed, values were averaged and expressed as the mean ± standard deviation. NO inhibition rates were expressed as a percent relative to the amount of NO evolved after exposure to LPS. When the letters are not same, values are significantly different ($p < 0.05$) (Tukey's test).

the former was further purified using silica gel chromatography. Eight fractions were recovered (Figure 2). When the fractions were applied at 25 μg/mL, NO reduction rates of 22.6−70.5% were obtained. Of these, the AF fraction showed the highest reduction rate, being comparable to that of L-NMMA.

The polyphenol compounds of the eight fractions (AA−AH) were analyzed (Table 3). AF, which showed the highest total polyphenol content, included eight polyphenol compounds (in the highest amount order, 3,4-dihydroxybenzoic acid, quercetin, myricetin, catechin, ferulic acid, caffeic acid, syrigic acid, and *p*-coumaric acid). 3,4-Dihydroxybenzoic acid accounted for 37.6% of the total. AC and AB followed with the next highest total content. Although the highest anti-inflammatory activity was observed for AF with the highest total polyphenol content, AD and AG showed the second highest activity values despite low amounts of polyphenol compounds. Gallic acid and ellagic acid are major components of AD and AG, respectively. The activity patterns of the fractions (Figure 2) did not agree with the patterns of total polyphenol content (Table 3).

Inhibitory Effect of the AF Fraction on Ear Edema in Mice. The inhibitory effect of the AF fraction on edema in mice was evaluated. As shown in Table 4, topical application of TPA to mice caused 0.312 mm of ESR. However, when AF was applied at 10 μg/20 μL to TPA-treated ears (AF-10), the ESR value was decreased to 0.112 mm, corresponding to a 63.4% reduction in the rate. As the AF content increased to 20 μg/20 μL (AF-20), the reduction rate further increased to 85.7%. This value was lower than for indomethacin (90.4%), which is a commercial product.

Inhibitory Effect of the AF Fraction on Writhing Response in Mice. A response test using acetic acid is usually used to evaluate the writhing response of the central and peripheral nervous systems.[20] After writhing response was induced in mice via abdominal injection of acetic acid, AF was administered orally. As shown in Figure 3, when no supplement was given (control), the writhing response value was 34.6.

Table 3. Contents of Polyphenol Compounds in Various Fractions Isolated from Juice−Pulp−Seed Wine[a]

	fractions							
polyphenol (g/kg)	AA	AB	AC	AD	AE	AF	AG	AH
gallic acid	2.13 ± 0.23	–	–	14.15 ± 0.11	–	–	0.99 ± 0.17	–
3,4-dihydroxybenzoic acid	–	–	2.04 ± 0.10	2.27 ± 0.05	–	57.54 ± 1.10	2.12 ± 0.15	–
catechin	–	1.36 ± 0.10	–	–	–	18.16 ± 0.89	0.55 ± 0.06	0.86 ± 0.15
caffeic acid	–	–	–	–	–	9.06 ± 0.27	–	–
epicatechin	–	1.85 ± 0.06	2.44 ± 0.10	0.82 ± 0.10	–	–	–	–
p-coumaric acid	–	3.40 ± 0	–	–	0.51 ± 0.05	2.04 ± 0.25	–	–
ellagic acid	–	–	106.65 ± 1.27	2.25 ± 0.06	8.35 ± 0.44	–	52.75 ± 0.40	19.29 ± 0.36
myricetin	–	43.19 ± 1.11	4.32 ± 0.23	–	–	25.83 ± 0.45	–	–
quercetin	–	10.19 ± 0.66	20.37 ± 0.87	–	–	25.91 ± 0.40	0.41 ± 0.03	–
kaempferol	–	–	–	–	–	–	–	–
malvidin 3-glucoside	–	24.80 ± 1.54	–	–	–	–	–	1.59 ± 0.10
rutin	–	14.04 ± 0.84	–	3.27 ± 0.20	–	–	–	–
p-hydroxybenzoic acid	–	8.63 ± 1.03	–	–	–	–	–	–
ferulic acid	–	–	–	–	–	9.86 ± 0.51	–	–
chlorogenic acid	–	2.51 ± 0.08	0.99 ± 0.14	–	0.55 ± 0.04	–	2.38 ± 0.17	1.87 ± 0.09
syrigic acid	–	–	–	–	–	4.59 ± 0.22	–	–
ethyl gallate	–	–	–	–	–	–	1.14 ± 0.07	–
total	2.13	109.97	136.81	22.76	9.41	152.99	60.34	23.61

[a]Identical experiments were three times repeated and expressed as the mean ± standard deviation. −, not detected.

Table 4. Anti-inflammatory Effect of the AF Fraction on TPA-Induced Ear Edema in Mice

treatment[a]	dose	*N*[b]	before[c] (mm)	after[c] (mm)	ESR[c,d] (mm)
vehicle		10	0.21 ± 0.01	0.22 ± 0.01	0.01 ± 0.01
TPA		12	0.19 ± 0.03	0.51 ± 0.02	0.31 ± 0.10c
AF-10	10 μg/ear	12	0.18 ± 0.01	0.30 ± 0.02	0.11 ± 0.01b
AF-20	20 μg/ear	12	0.18 ± 0.01	0.23 ± 0.01	0.05 ± 0.01a
INDO	10 μg/ear	12	0.18 ± 0.01	0.23 ± 0.02	0.04 ± 0.01a

[a]After TPA in acetone was topically applied, mice were treated for 30 min with AF-10, AF-20, and indomethacin (INDO). Vehicle, acetone; TPA, TPA + acetone; AF-10, 10 μg AF/20 μL acetone; AF-20, 20 μg AF/20 μL acetone. [b]*N*, number of animals. [c]Data are shown as the mean ± SD. When the letters are not same, values are significantly different ($p < 0.05$) (Tukey's test). [d]ESR (ear swelling response): difference between ear thickness before and after challenge.

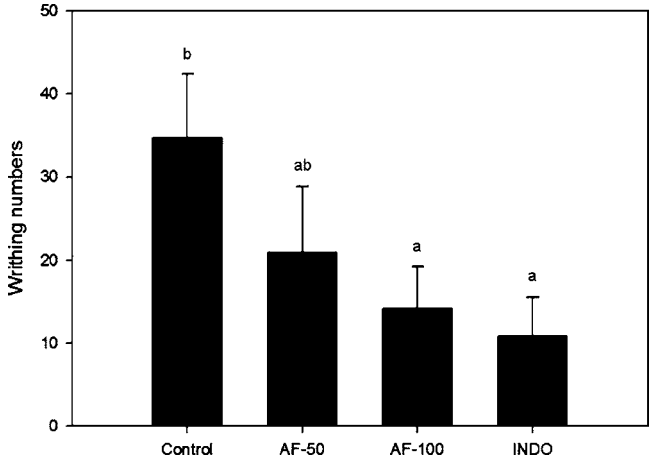


Figure 3. Inhibitory effect of the AF fraction on acetic acid-induced writhing response in mice. Mice were orally administered AF-10, AF-20, and indomethacin (INDO) before an intraperitoneal injection of acetic acid. Control, acetic acid + saline solution; AF-50, acetic acid + 50 mg AF/kg-mouse; AF-100, acetic acid + 100 mg AF/kg-mouse; INDO, acetic acid + 20 mg indomethacin/kg-mouse. After three independent experiments were performed, values were averaged and expressed as the mean ± standard deviation. When the letters are not the same, values are significantly different ($p < 0.05$) (Tukey's test).

However, when AF was supplied as a supplement at 50 and 100 mg/kg (AF-50 and AF-100), respectively, the response numbers were reduced by 39.7 and 59.2%. When indomethacin, an anti-inflammatory drug, was supplied as a supplement, the response number was decreased to 10.0, corresponding to a 68.6% reduction in the rate.

Inhibitory Effect of the AF Fraction on Vascular Permeability in Mice. Some disorders in blood tissues can be caused by a stimulus from an inflammation reaction, resulting in changes in the inner membrane of vein cells. The degree of inflammation in mice induced by oral intake of acetic acid was determined on the basis of the permeability of veins. The supplementation effect of AF on mice vascular permeability was investigated. As shown in Figure 4, permeability reduction rates of samples were calculated relative to the control, for which a saline solution was added, and its value was set to zero. When AF was applied at 50 and 100 mg/kg (AF-50 and AF-100), corresponding inhibitory values were 45.6 and 61.7%. Indomethacin (INDO) showed a value of 72.6%. Thus, AF from Korean black raspberry wine suppresses damage of the vein.

DISCUSSION

Among three types of Korean black raspberry wines, the juice−pulp−seed wine contained the largest amounts of most of the key polyphenol compounds due to supplementation with

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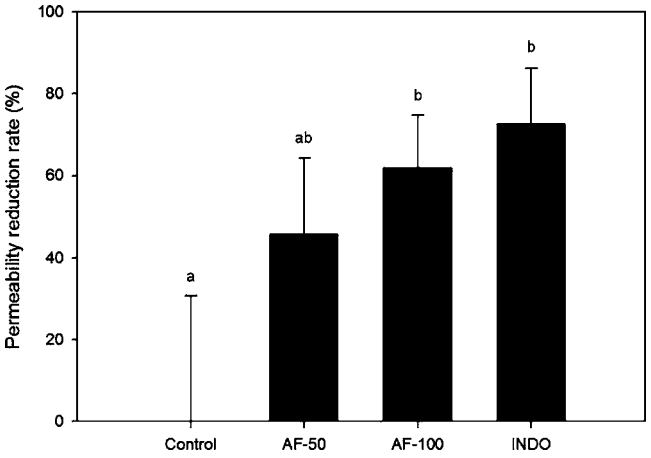

Figure 4. Inhibitory effect of the AF fraction on acetic acid-induced vascular permeability in mice. Mice were orally administered AF-10, AF-20, and indomethacin (INDO) before an intraperitoneal injection of acetic acid. Control, acetic acid + saline solution; AF-50, acetic acid + 50 mg AF/kg-mouse; AF-100, acetic acid + 100 mg AF/kg-mouse; INDO, acetic acid + 20 mg indomethacin/kg-mouse. The vascular permeability was expressed as the amount of total dye that leaked into the intraperitoneal cavity. Inhibition rates were expressed as a reduction percent relative to the absorbance after exposure to acetic acid. After three independent experiments were performed, values were averaged and expressed as the mean ± standard deviation. When the letters are not the same, values are significantly different ($p < 0.05$) (Tukey's test).

raspberry seeds (Table 2). Amounts of the polyphenol compounds in Korean black raspberry wines were, in most cases, higher than those in commercial grape wines.[21−24] Polyphenol compounds in our juice−pulp−seed wine were in the highest amount order of 3,4-dihydroxybenzoic acid, epicatechin, malvidin 3-glucoside, rutin, catechin, and gallic acid at 55−84 mg/L (Table 2). On the other hand, in commercial grape wines, the key polyphenol compounds consist of catechin, epicatechin, and gallic acid at 13−42 mg/L and quercetin at 3−5 mg/L.[21−24] Approximately, our juice−pulp−seed wine contained 6−50 times more 3,4-dihydroxybenzoic acid and 3−13 times more catechin, epicatechin, and quercetin. The major compound, 3,4-dihydroxybenzoic acid, is also found in grape wines but not as the major component (2−4 mg/L).[21,24] Our juice−pulp−seed wine contained more catechin, which accounts for the largest amount of polyphenol compounds in grape wines.[21−23]

For Korean black raspberry wines, the anti-inflammatory activity is apparently related to the total content of polyphenol compounds, considering that the juice−pulp−seed wine with largest amounts of most of the key polyphenol compounds (Table 2) showed the highest anti-inflammatory activity (Table 1). Similarly for purified fractions (AA−AH), the AF fraction with highest total polyphenol amount (Table 3) showed the highest anti-inflammatory activity (Figure 2). However, the amounts of major polyphenol compounds rather than their total seemed to determine the activity order. Among the polyphenol compounds, 3,4-dihydroxybenzoic acid is considered to be important for its anti-inflammatory activity because it is the most abundant polyphenol compound in the AF fraction. The AD and AG fractions with the second and third highest activity levels also have considerable amounts of 3,4-dihydroxybenzoic acid. In this respect, 3,4-dihydroxybenzoic acid is probably the most important component for the anti-inflammatory effect in Korean black raspberry wines. On the other hand, considering that the major polyphenol compound of the AC and AG fractions was ellagic acid (Table 3) and their anti-inflammatory activities were lower than that of the AF fraction, 3,4-dihydroxybenzoic acid is apparently better than ellagic acid in anti-inflammatory activity. Similarly, the activity of 3,4-dihydroxybenzoic acid is higher than that of myricetin from comparison between the AF and AB fractions. The strong anti-inflammatory effect of black raspberry wines, similarly for grape wines, is considered to be exerted by a combination of various polyphenol compounds rather than any one compound. Black raspberry wines probably have higher anti-inflammatory activities than grape wines due to larger amounts of key polyphenol compounds.

Inhibitory effects of our black raspberry wine were achieved in mice, for example, for ear edema, writhing response, and vascular permeability (Table 4; Figures 3 and 4). The effects were comparable to those of indomethacin, a well-known anti-inflammatory drug. Polyphenol compounds are known to have various anti-inflammatory activities. 3,4-Dihydroxybenzoic acid, which was a major compound of our juice−pulp−seed wine, has a considerable inhibitory activity for TPA-induced edema in mice,[25] and it decreases the production of inflammatory cytokines in the heart and kidney in mice.[26] Besides, catechin showed anti-inflammatory effect by reducing the expression of IL-6 and IL-8 in human dental pulp cells.[27] Epicatechin inhibits TPA-induced O_2 generation, whereas quercetin and kaempferol inhibit cytokine-induced expression of iNOS and COX-2.[28] Gallic acid is effective in significantly reducing paw edema.[29] The inhibitory activities of the black raspberry wine are considered to be contributed by a combination of the effects that were exerted by polyphenol compounds.

In conclusion, the amounts of key polyphenol compounds were increased during alcoholic fermentation of Korean black raspberries. The amounts of these compounds in wines were enhanced by supplementation with pulp and seeds. Black raspberry wines showed various anti-inflammatory activities in tests using both cells and mice, and among polyphenol compounds, 3,4-dihydroxybenzoic acid was considered to be a key compound.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Heo, S. J.; Lee, D. Y.; Choi, H.K..; Lee, J. H.; Kim, J. H.; Cho, S. M.; Lee, H. J.; Auh, J. H. Metabolite fingerprinting of *bokbunja* (*Rubus coreanus* Miquel) by UPLC-qTOF-MS. *Food Sci. Biotechnol.* **2011**, *20*, 567−570.

(2) Kim, T. J. *Korean Resources Plants II*; Publishing Department of Seoul National University: Seoul, Korea, 1996; p 140.

(3) Ku, C. S.; Mun, S. P. Antioxidant activities of ethanol extracts from seeds in fresh *Bokbunja* (*Rubus coreanus* Miq.) and wine processing waste. *Bioresour. Technol.* **2008**, *99*, 4503−4509.

(4) Lim, J. W.; Jeong, J. T.; Shin, C. S. Compound analysis and sensory evaluation of Korean black raspberry (*Rubus coreanus* Miquel) wines. *Int. J. Food Sci. Technol.* **2012**, *47*, 918−926.

(5) Jakobek, L.; Seruga, M.; Seruga, B.; Novak, I.; Medvidovic-Kosanovic, M. Phenolic compound composition and antioxidant

activity of fruits of *Rubus* and *Prunus* species from Croatia. *Int. J. Food Sci. Technol.* **2009**, *44*, 860−868.
(6) Wu, X.; Pittman, H. E., III; Hager, T.; Hager, A.; Howard, L.; Prior, R. L. Phenolic acids in black raspberry and in the gastrointestinal tract of pigs following ingestion of black raspberry. *Mol. Nutr. Food Res.* **2009**, *53*, S76−S84.
(7) Yang, H. M.; Oh, S. M.; Lim, S. S.; Shin, H. K.; Oh, Y. S.; Kim, J. K. Antiinflammatory activities of *Rubus coreanus* depend on the degree of fruit ripening. *Phytother. Res.* **2008**, *22*, 102−107.
(8) Ju, H. K.; Cho, E. J.; Jang, M. H.; Lee, Y. Y.; Hong, S. S.; Park, J. H.; Kwon, S. W. Characterization of increased phenolic compounds from fermented Bokbunja (*Rubus coreanus* Miq.) and related antioxidant activity. *J. Pharm. Biomed. Anal.* **2009**, *49*, 820−827.
(9) Yang, H. M.; Lim, S. S.; Lee, Y. S.; Shin, H. K.; Oh, Y. S.; Kim, J. K. Comparison of the anti-inflammatory effects of the extracts from *Rubus coreanus* and *Rubus occidentalis*. *Korean J. Food Sci. Technol.* **2007**, *39*, 342−347.
(10) Jeon, S. K.; Lee, J. W.; Lee, I. S. Effect of antioxidant activity and induction of DNA damage on human gastric cancer cell by *Rubus coreanus* Miquel. *J. Life Sci.* **2007**, *17*, 1723−1728.
(11) Lala, P. K.; Chakraborty, C. Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol.* **2001**, *2*, 149−156.
(12) Lee, S. H.; Lee, Y. P.; Kim, S. Y.; Jeong, M. S.; Lee, M. J.; Kang, H. W.; Jeong, H. J.; Kim, D. W.; Sohn, E. J.; Jang, S. H.; Kim, Y. H.; Kwon, H. J.; Cho, S. W.; Park, J. S.; Eum, W. S.; Choi, S. Y. Inhibition of LPS-induced cyclooxygenase 2 and nitric oxide production by transduced PEP-1-PTEN fusion protein in RAW 264.7 macrophage cells. *Exp. Mol. Med.* **2008**, *40*, 629−638.
(13) Liu, K. L.; Chen, H. W.; Wang, R. Y.; Lei, Y. P.; Sheen, L. Y.; Lii, C. K. DATS reduces LPS-induced iNOS expression, NO production, oxidative stress, and NK-κB activation in RAW 264.7 macrophages. *J. Agric. Food Chem.* **2006**, *54*, 3472−3478.
(14) Institute of Laboratory Animal Resource. *Guide for the Care and Use of Laboratory Animals*; National Academies Press: Washington, DC, 2001; pp 11−40.
(15) Cantos, E.; Garcia-Viguera, C.; de Pascual-Teresa, S. A.; Tomas-Barberan, F. A. Effect of postharvest ultraviolet irradiation on resveratrol and other phenolics of cv. Napoleon table grapes. *J. Agric. Food Chem.* **2000**, *48*, 4606−4612.
(16) Cheon, M. S.; Yoon, T.; Yasukawa, K.; Yu, S. Y.; Kim, S. J.; Choi, G.; Moon, B. C.; Lee, A. Y.; Choo, B. K.; Kim, H. K. Effects of aralia continentalis and angelica biserrata on Inflammatory response in lipopolysaccharide-Induced RAW 264.7 macrophages and phorbol ester-induced ear edema. *J. Korean Soc. Appl. Biol. Chem.* **2009**, *52*, 157−162.
(17) Olufunke, D. M.; Oladosu, I. A.; Adeleke, O.; Ali, M. S. Chemical composition and anti-inflammatory activity of the essential oil of the aerial part of *Mezoneuron benthamianum* Baill. (Caesalpinoideae). *Eur. J. Appl. Sci.* **2009**, *1*, 30−33.
(18) Koster, R.; Anderson, M.; de Beer, E. J. Acetic-acid for alagesic screening. *Fed. Proc.* **1959**, *18*, 412−418.
(19) Khanna, M.; Chaturvedi, U. C.; Sharma, M. C.; Pandey, V. C.; Mathur, A. Increased capillary permeability mediated by a dengue virus-induced lymphokine. *Immunology* **1990**, *69*, 449−453.
(20) Siegmund, E.; Cadmus, R.; Lu, G. A method for evaluating both non-narcotic and narcotic analgesics. *Proc. Soc. Exp. Biol.* **1957**, *95*, 729−731.
(21) Saenz-Navajas, M. P.; Tao, Y. S.; Dizy, M.; Ferreira, V.; Fernandez-Zurbano, P. Relationship between nonvolatile composition and sensory properties of premium Spanish red wines and their correlation to quality perception. *J. Agric. Food Chem.* **2010**, *58*, 12407−12416.
(22) Martin, F.; Alvaro, P. N.; Viviana, J.; Mariela, A.; Fernando, Z. Phenolic characterization of Malbec wines from Mendoza Province (Argentina). *J. Agric. Food Chem.* **2010**, *58*, 2388−2397.
(23) Cabrita, M. J.; Torres, M.; Palma, V.; Alves, E.; Patao, R.; Costa Freitas, A. M. Impact of malolactic fermentation on low molecular weight phenolic compounds. *Talanta* **2008**, *74*, 1281−1286.
(24) Rodriguez-Delgado, M. A.; Malovana, S.; Perez, J. P.; Borges, T.; Garcia Montelongo, F. J. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J. Chromatogr., A* **2001**, *912*, 249−257.
(25) Rasadah, M. A.; Farediah, A.; Wong, C. L. Anti-inflammatory activity of extracts and compound from *Vitex Negundo*. *J. Trop. For. Sci.* **2005**, *17*, 481−487.
(26) Lin, C.-Y.; Huang, C.-S.; Huang, C.-Y.; Yin, M.-C. Anti-coagulatory, antiinflammatory, and antioxidative effects of protocatechuic acid in diabetic mice. *J. Agric. Food Chem.* **2009**, *57*, 6661−6667.
(27) Nakanishi, T.; Mukai, K.; Yumoto, H.; Hirao, K.; Hosokawa, Y.; Matsuo, T. Anti-inflammatory effect of catechin on cultured human dental pulp cells affected by bacteria-derived factors. *Eur. J. Oral Sci.* **2010**, *118*, 145−150.
(28) Pan, M.-H.; Lai, C.-S.; Dushenkov, S.; Ho, C.-T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467−4477.
(29) Corbett, S.; Drayton, R.; Steinbardt, R. Evaluation of the anti-inflammatory effects of ellagic acid. *J. Perianesth. Nurs.* **2010**, *25*, 214−220.