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Inhibitory Effects of Anthocyanins and Other Phenolic Compounds on Nitric Oxide Production in LPS/IFN-γ-Activated RAW 264.7 Macrophages

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Flavonoids have been reported to lower oxidative stress and possess beneficial effects on cardiovascular diseases and chronic inflammatory diseases associated with nitric oxide (NO). Common phenolic compounds, including phenolic acids, flavonols, isoflavones, and anthocyanins, present in fruits were investigated for their effects on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages. Phenolic compounds at the range of 16−500 μM that inhibited NO production by >50% without showing cytotoxicity were the flavonols quercetin and myricetin, the isoflavone daidzein, and the anthocyanins/anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides. Anthocyanins had strong inhibitory effects on NO production. Anthocyanin-rich crude extracts and concentrates of selected berries were also assayed, and their inhibitory effects on NO production were significantly correlated with total phenolic and anthocyanin contents. This is the first study to report the inhibitory effects of anthocyanins and berry phenolic compounds on NO production.

KEYWORDS: Nitric oxide; RAW 264.7 macrophages; flavonols; isoflavones; anthocyanins; anti-inflammation; cytotoxicity; berries; functional foods; nutraceuticals

INTRODUCTION

Flavonoids including anthocyanins (**Figure 1**) are polyphenolics occurring in a wide range of plants, with >8000 chemically distinct compounds identified (*1*). Flavonoids such as quercetin occur in nearly all common fruits and vegetables, and the average daily intake of flavonoids, expressed as aglycons, is estimated at a few hundred milligrams per day (*2*). Several flavonoids have been shown to exhibit antioxidant, anti-inflammatory, anticarcinogenic, and estrogenic activities, to inhibit enzymes, and to prevent coronary heart disease (*2*−*5*).

Nitric oxide (NO) is a diatomic free radical produced from L-arginine by constitutive and inducible nitric oxide synthase (cNOS and iNOS) in numerous mammalian cells and tissues. It was first identified as an endothelium-derived relaxation factor and is now recognized as a regulator of many mammalian cell and tissue functions (*6*, *7*). There are three distinct isoforms of nitric oxide synthases (NOSs). The endothelial nitric oxide synthase (eNOS) is present in endothelium, which is known to play an important role in the dynamic control of vascular tone. The neural nitric oxide synthase (nNOS) is mainly present in neural tissues and serves as a neurotransmitter. eNOS and nNOS are constitutive isoforms of nitric oxide synthases and are also known as cNOS. The third nitric oxide synthase (iNOS) is induced by either bacterial lipopolysaccharide (LPS) or a number of cytokines including tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) in macrophages, hepatocytes, and endothelial cells. iNOS and nitric oxide are involved in host defense and immunity and modulate the inflammatory response (*8*, *9*). Nitric oxide (NO), superoxide (O^{2-}), and their reaction product peroxynitrite ($ONOO^-$) may be generated in excess during the host response against viral and bacterial infections and contribute to some pathogenesis by promoting oxidative stress, tissue injury, and even cancer (*10*, *11*).

Recently the effects of selected flavonoids on NO production in LPS/IFN-γ-activiated RAW 264.7 macrophage cells have been reported. These studies have shown that some flavonoids or methanol extracts of plants inhibit NO production. Published works have investigated extracts of *Ginkgo biloba* (*12*), edible Japanese plants (*13*), theaflavin-3,3′-digallate and epigallocatechin-3-gallate from tea (*14*), quercetin and resveratrol from wine (*15*), coumarins from fruits of *Citrus hystrix* DC and *Citrus limon* (*16*, *17*), extracts rich in procyanidins from pine bark (*18*, *19*), and flavanones, flavones, isoflavones, and flavonols (*20*, *21*). The results of these investigations indicate possible protective effects of polyphenolics against cardiovascular disease and chronic inflammatory diseases. The proposed mechanisms associated with the reduction in NO production are scavenging

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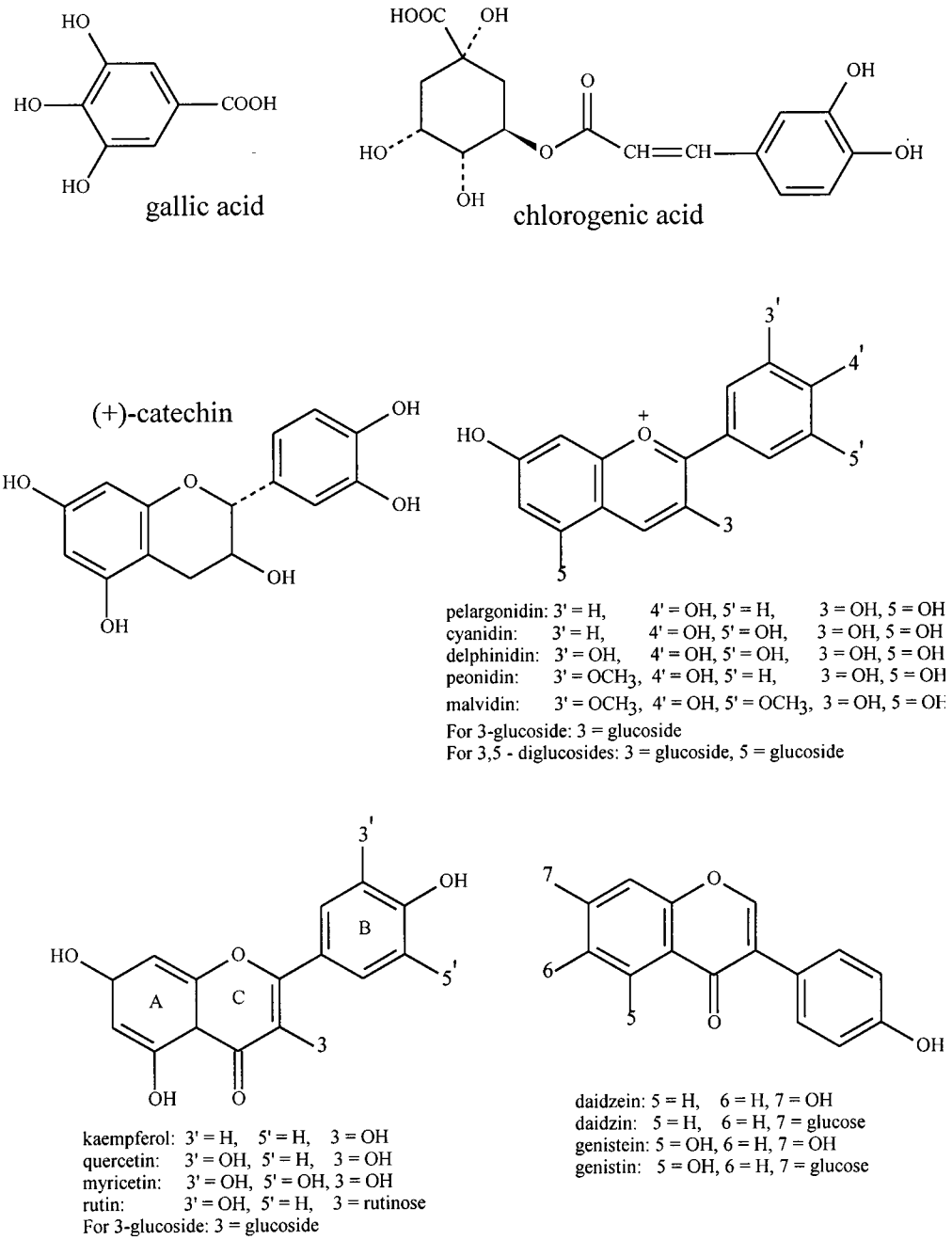

Figure 1. Structures of phenolic compounds.

of NO radicals, direct inhibition of iNOS enzyme activity, and/or inhibition of iNOS gene expression (*19, 21*).

The objectives of the present study were (1) to study effects of 23 commonly occurring phenolic compounds, including 9 anthocyanidins/anthocyanins, 7 flavonols, and 4 isoflavones, on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages and (2) to relate the NO inhibitory activities of pure phenolic compounds to the activities of anthocyanin-rich crude extracts and their concentrates from four types of berries.

MATERIALS AND METHODS

Materials and Reagents. Recombinant mouse IFN-γ was obtained from BD PharMingen (San Diego, CA). Lipopolysaccharides (LPS, by phenolic extraction from *Salmonella enteritidis*), (+)-catechin, sodium nitrite, *N*-(1-naphthyl)ethylenediamine dihydrochloride, sulfanilamide, phosphoric acid, myricetin, dimethyl sulfoxide (DMSO), chlorogenic acid, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) were from Sigma Chemical Co. (St. Louis, MO). Gallic acid and rutin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dulbecco's modified Eagle medium (DMEM), penicillin−streptomycin, glutamine, sodium pyruvate, and fetal bovine serum (FBS) were obtained from Life Technologies (Gaithersburg, MD). Quercetin, quercetin 3-glucoside (isoquercitrin), kaempferol, kaempferol 3-glucoside, kaempferol 3-rutinoside, daidzein, daidzin, genistein, genistin, cyanidin chloride, cyanidin 3-glucoside (kuromanin) chloride, cyanidin 3,5-diglucoside (cyanin) chloride, delphinidin chloride, malvidin chloride, malvidin 3-glucoside (oenin) chloride, malvidin 3,5-diglucoside (malvin) chloride, pelargonidin chloride, and peonidin chloride were from Extrasynthese (Genay, France).

Extraction and Purification of Phenolic Compounds from Berries. Phenolic compounds were extracted from frozen samples of Saskatoon berries (*Amelanchier alnifolia* Nutt.), blueberries, blackberries, and black currants. Saskatoon berries (cv. Smoky) were obtained from the Berry Basket (Clairmont, AB, Canada). Black currants were obtained from Riverbend Country Gardens (Sylvan Lake, AB, Canada). Frozen blackberries and blueberries were purchased from a local supermarket.

For the extraction of phenolic compounds, 100 g of berries was combined with 400 mL of 80% ethanol in water in a Waring blender and blended for 5 min. The mixture was then filtered through a Whatman No. 4 filter paper in a Büchner funnel. Ethanol was removed using a rotary evaporator under vacuum at 35 °C, and extracts were freeze-dried. The freeze-dried samples, namely crude extracts, were tested for their effects on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages and/or were purified for further studies.

In addition to anthocyanins and other phenolic compounds, the crude extracts contained carbohydrates, proteins, minerals, and other fruit compounds. To study the effects of phenolic compounds present in berries on NO production, further purification was necessary to remove these impurities. The crude extracts were purified using a column (40 × 3 cm i.d.) packed with the nonionic hydrophobic polystyrene polymer Amberlite XAD 16HP (Rohm and Haas Canada, Inc., ON, Canada). Note that Amberlite XAD 16HP meets the requirements of FDA 21 CFR 173.65 for the removal of organic substances from aqueous foods. It can therefore be used for recovery of anthocyanins, when the FDA regulation is applicable. The packing material was washed and conditioned according to the manufacturer's directions. However, before any sample was loaded, the column was conditioned with water containing 1% acetic acid. Freeze-dried crude extracts (10 g) were dissolved in 40 mL of 1% acetic acid, and the mixture was fully loaded onto the column at a flow rate of 3 mL/min. The column was washed using 1% acetic acid at a flow rate of 3 mL/min until sugar was not detected using an ATAGO hand refractometer (ATAGO Co.). The anthocyanins and other phenolic compounds were eluted with 80% ethanol in water containing 1% acetic acid at a flow rate of 3 mL/min. The colored eluents were collected and further concentrated using a rotary evaporator under vacuum at 35 °C to remove ethanol, followed by freeze-drying. These freeze-dried samples, namely concentrates, were studied for their effects on NO production and/or kept at −20 °C for further uses.

Measurement of Phenolic Compounds. The crude extracts and concentrates of berries were analyzed for their total phenolics, tartaric esters, flavonols, and anthocyanins contents using the spectrophotometric method of Fukumoto and Mazza (*22*). The method consisted of mixing 0.25 mL of methanol-dissolved sample with 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of double-deionized water with 2% HCl. The absorbance of the solution was read at 280, 320, 360, and 520 nm, respectively. Standards used were chlorogenic acid, caffeic acid, quercetin, and cyanidin for total phenolics, tartaric esters, flavonols, and anthocyanins, respectively. All samples and standards were prepared in 80% methanol in water, and quercetin was prepared in methanol.

Cell Culture. The mouse monocyte/macrophage cell line RAW 264.7 [American Type Culture Collection (ATCC), Manassas, VA] was cultured in DMEM (phenol red free) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, streptomycin (100 μg/mL), and penicillin (100 units/mL) (*23*). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell number was assessed by trypan blue dye exclusion on a Neubauer hemacytometer. Cells were grown to 90% confluence in sterile cell culture flasks and gently detached using a scraper (Fisher Scientific, Pittsburgh, PA). For phenolic compound treatment tests, cells were cultured in triplicate in Costar flat-bottom cell culture plates (Corning Inc., Ithaca, NY). Cells were plated at a density of 6×10^5 cells/well in 24-well cell culture plates and grown for ~1 h to allow them to attach to the plate. Compounds to be tested were initially dissolved in 10 μL of DMSO, and then DMEM was added to make solutions in a series of concentrations with a dilution factor of 2. The final concentrations of test compound that cells received were 16, 31, 63, 125, 250, and 500 μM, respectively. For berry crude extracts and concentrates, the final concentrations were 16, 31, 63, 125, 250, and 500 μg of product/mL, respectively. Cells were supplemented with the test compounds for 1 h before stimulation with 10 ng/mL LPS and 10−50 units/mL IFN-γ. The activated cells were further incubated for 24 h (*19*, *23*, *24*). Then supernatants were collected to determine nitrite concentration and/or stored at −80 °C for further use. Control cells were grown under identical conditions but were not exposed to the test compounds or LPS/IFN-γ.

NO Determination. Nitrite concentration was used as an indication of NO production. The procedure for NO determination was based on the Griess reaction (*25*). One hundred microliters of culture supernatant or sodium nitrite standard (5.2−103.6 μM) was mixed with an equal volume of Griess reagent [a mixture of 0.1% (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid; the two parts being mixed together within 1 h of use] using a 96-well plate. After 20 min at room temperature, the absorbance at 540 was measured by a microtitration plate reader (Molecular Devices, Spectramax Plus 384, Sunnyvale, CA).

Analysis of nitrite in supernatants containing anthocyanins was conducted as described by Wang and Mazza (*26*). In brief, 100 μL of cell culture supernatant was mixed with an equal volume of Griess reagent as above using a 96-well microtitration plate; a set of parallel analyses was also conducted by applying only 100 μL of 2.5% (v/v) phosphoric acid instead of Griess reagent to each of 100 μL of cell supernatants or the mixture of standard sodium nitrite and cyanidin chloride. After 20 min at room temperature, the absorbance at 540 nm was measured using the microtitration plate reader. The net absorbance of the product of Griess reaction was obtained by subtracting that of anthocyanins from the total. The absorbance was referred to a nitrite standard curve to determine the nitrite concentration in supernatants.

Cell Viability. Cell viability was determined by the MTT assay (*27*) and/or the resazurin-based in vitro toxicology assay kit, TOX-8 (Sigma Chemical Co.). Briefly, when the MTT assay was used, MTT (5 mg/mL) dissolved in DMEM without phenol red was filtered through a 0.2 μm filter and stored at 2−8 °C for routine use. This stock solution was diluted 10-fold with DMEM for each culture being assayed, and then 0.5 mL of the MTT (0.5 mg/mL) solution was added to each well of a 24-well plate. Note that after culture supernatants were collected and before MTT solution was added, culture wells were washed once with 1 mL of phosphate-buffered saline (PBS) to remove the remained test compounds. After incubation for an additional 4 h at 37 °C in a 5% CO_2 incubator, media were removed and the converted dye, formazan, was solubilized with 0.3 mL of acidified 2-propanol (0.04−0.1 N HCl in 2-propanol). One hundred and fifty microliters of the 2-propanol solution was transferred to a 96-well plate, and the absorbance was read at a wavelength of 570 nm with a reference wavelength of 630 nm.

A resazurin-based in vitro toxicology assay kit, namely, the resazurin assay, was used when tested compounds, such as anthocyanins, interfered with the detection of formazan as described above. The resazurin dye solution (0.5 mL), 10 times diluted from its original stock solution, was added to each well of a 24-well plate. After incubation for an additional 4 h at 37 °C and 5% CO_2, 200 μL of the supernatant with the bioreduced fluorescent resazurin intermediate (purple-red color) was transferred to a 96-well plate, and the absorbance was measured at 570 nm.

The viability of cells activated only by LPS/IFN-γ was arbitrarily set as 100, and all other viabilities, that is, cells receiving different treatments but within the same 24-well plate, were normalized to that of the LPS/IFN-γ-activated control cells.

Statistics. Means, standard deviations, and regression coefficients were calculated using Statistical Analysis System (SAS for Windows v 8). Mean comparisons were made with the least significant difference (LSD) test at a 0.05 significant level.

RESULTS AND DISCUSSION

When LPS/IFN-γ was administered to RAW 264.7 macrophages, NO production, measured as nitrite, increased dramatically from the basal level of ~2 to ≥40 μM after 24 h. To determine the effects of anthocyanins and other phenolic compounds on NO production, different concentrations of these compounds (500, 250, 125, 63, and 16 μM) were incubated with the LPS/IFN-γ-activated RAW 264.7 macrophages. Cell viability was assayed to exclude the possibility that the inhibitory effects of phenolic compounds were due to their cytotoxicity. The MTT assay was used for the colorless phenolic compound-treated macrophages, whereas the resazurin assay was used for antho-

Table 1. Effects of Phenolic acids, Catechins, Flavonols, and Isoflavones on Nitric Oxide Production and Viability of LPS/IFN-γ-Activated RAW 264.7 Macrophages

concn (μM)	compound	nitrite[a] (μM)	cell viability[b] (%)	compound	nitrite (μM)	cell viability (%)	compound	nitrite (μM)	cell viability (%)
control[c]	gallic acid	1.8[g] (0.1)		chlorogenic acid	1.8[c] (0.1)		(+)-catechin	1.9[d] (0.2)	
LPS/IFN-γ[d]		46.2[cd] (1.5)	100 (0)		46.2[b] (1.5)	100 (0)		39.7[a] (1.5)	100 (0)
16[e]		49.7[b] (0.3)	110 (11)		48.3[ab] (1.2)	117 (10)		39.4[a] (0.4)	98 (10)
31		53.6[a] (3.8)	108 (12)		48.9[a] (3.4)	114 (16)		37.5[b] (0.4)	107 (12)
63		46.9[c] (1.1)	108 (16)		46.7[ab] (0.7)	111 (6)		37.6[b] (0.7)	118 (5)
125		43.8[d] (0.4)	97 (4)		46.2[b] (0.4)	113 (15)		36.2[bc] (1.2)	102 (5)
250		34.0[e] (1.4)	108 (15)		47.5[ab] (1.0)	117 (13)		35.1[c] (1.0)	108 (10)
500		13.4[f] (0.2)	20 (1)		46.7[ab] (1.3)	100 (6)		35.5[c] (0.3)	126 (11)
control	kaempferol	1.7[g] (0.1)		kaempferol	1.7[e] (0.1)		kaempferol	1.9[c] (0.2)	
LPS/IFN-γ		49.6[a] (0.2)	100 (0)	3-glucoside	49.6[a] (0.2)	100 (0)	3-rutinoside	39.7[a] (1.5)	100 (0)
16		35.7[b] (1.0)	117 (11)		49.0[a] (2.2)	111 (16)		40.7[a] (1.3)	114 (4)
31		27.5[c] (1.0)	89 (9)		49.3[a] (0.4)	108 (20)		39.2[a] (2.1)	112 (13)
63		18.7[d] (0.4)	53 (15)		48.2[ab] (0.2)	107 (12)		39.4[a] (2.3)	108 (2)
125		13.5[e] (0.4)	27 (4)		47.1[bc] (0.4)	98 (9)		38.2[a] (1.7)	109 (11)
250		12.0[f] (1.3)	19 (5)		46.0[c] (1.1)	95 (10)		33.8[b] (1.1)	111 (11)
500		11.3[f] (0.4)	18 (7)		41.2[d] (0.4)	101 (16)		31.7[b] (1.5)	85 (10)
control	quercetin	1.7[h] (0.0)		quercetin	2.2[f] (0.2)		quercetin	2.2[f] (0.2)	
LPS/IFN-γ		44.8[a] (2.0)	100 (0)	3-glucoside	44.4[a] (1.9)	100 (0)	3-rutinoside	44.4[a] (1.9)	100 (0)
16		40.9[b] (1.0)	114 (15)		43.6[a] (1.2)	117 (6)		43.8[ab] (0.9)	107 (8)
31		32.2[c] (0.7)	119 (5)		41.0[b] (0.8)	111 (13)		41.6[b] (1.7)	112 (7)
63		24.4[d] (1.2)	114 (3)		40.1[b] (0.7)	113 (10)		41.2[b] (0.4)	99 (11)
125		19.6[e] (0.5)	114 (10)		35.0[c] (0.7)	113 (8)		38.0[c] (3.4)	109 (8)
250		16.8[f] (0.3)	106 (11)		28.5[d] (0.6)	111 (11)		34.4[d] (0.2)	100 (6)
500		10.5[g] (0.5)	79 (12)		22.5[e] (0.5)	111 (8)		26.9[e] (0.9)	89 (14)
control	daidzein	1.8[g] (0.2)		daidzin	1.8[f] (0.2)		myricetin	1.9[g] (0.6)	
LPS/IFN-γ		39.3[a] (1.4)	100 (0)		39.3[a] (1.4)	100 (0)		58.1[ab] (1.7)	100 (0)
16		38.4[a] (1.4)	98 (11)		38.1[ab] (1.1)	113 (10)		59.3[a] (1.8)	107 (8)
31		36.2[b] (0.5)	108 (2)		36.5[bc] (1.2)	104 (4)		56.6[b] (1.3)	104 (11)
63		34.4[c] (0.6)	114 (1)		34.8[cd] (1.4)	108 (17)		53.3[c] (2.3)	100 (10)
125		27.6[d] (1.0)	107 (3)		33.9[d] (1.6)	101 (8)		42.8[d] (1.9)	102 (13)
250		19.4[e] (1.0)	76 (6)		31.5[e] (13)	52 (4)		21.8[e] (0.9)	118 (9)
500		13.0[f] (0.5)	33 (7)		30.9[e] (1.8)	62 (7)		7.7[f] (0.3)	74 (9)
control	genistein	1.7[g] (0.1)		genistin	1.7[f] (0.1)				
LPS/IFN-γ		37.1[b] (0.5)	100 (0)		37.1[ab] (0.5)	100 (0)			
16		40.3[a] (2.3)	92 (0)		39.2[a] (0.2)	103 (21)			
31		37.9[ab] (2.4)	99 (7)		38.6[a] (0.4)	88 (12)			
63		28.9[c] (2.4)	103 (3)		36.4[bc] (1.9)	108 (6)			
125		23.7[d] (2.1)	102 (6)		34.4[c] (0.5)	116 (16)			
250		13.7[e] (0.9)	21 (10)		28.4[d] (2.6)	130 (6)			
500		7.3[f] (0.5)	5 (1)		25.7[e] (0.7)	142 (8)			

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). Data for individual compounds with different letters denote significant difference in nitrite contents ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

cyanin-treated macrophages. The inhibitory effect (IE) was expressed as the percentage decrease of NO production as

$$\text{IE (\%)} = 100 - [\text{NO}]^{a}/[\text{NO}]^{b} \times 100$$

where $[NO]^a$ represents the NO concentration in supernatants from both phenolic compounds supplemented and LPS/IFN-γ-activated macrophages and $[NO]^b$ represents the NO concentration in supernatants from LPS/IFN-γ activated control macrophages.

Gallic Acid, Chlorogenic Acid, and (+)-Catechin. The inhibitory effect of gallic acid on NO production in LPS/IFN-γ-activated macrophages was dose-dependent (**Table 1**). The NO production decreased with the increase in concentration of gallic acid in the media. For example, the 125 and 250 μM gallic acid treatments attained 5 and 26% reduction in NO production, respectively. However, when concentration of the acid reached 500 μM, it displayed significant cytotoxicity toward the cells (**Table 1**). Chlorogenic acid, although a very strong antioxidant in vitro (*22*), showed no inhibitory effect on RAW 264.7 cells; instead, it may induce production of NO (**Table 1**). (+)-Catechin displayed a small but significant inhibitory effect compared to the LPS/IFN-γ control (**Table 1**). The NO production, expressed as nitrite concentration, was decreased by 11 or 12% for 250 or 500 μM treatments, respectively.

Flavonols. The inhibitory effect of seven flavonols on NO production in LPS/IFN-γ-activated macrophages is shown in **Table 1**. All test compounds demonstrated the dose-dependent inhibition. The results for kaempferol, however, clearly indicated that this compound exhibited marked cytotoxicity, which was observed with both the MTT and the resazurin assays. Quercetin and myricetin were able to inhibit NO production without significant cytotoxic effect. The $IC_{\geq 50}$ values, the concentration required to inhibit NO production by >50%, for quercetin and myricetin were 125 μM (IE = 56%) and 250 μM (IE = 62%), respectively. These inhibitory effects are similar to those reported by Kim et al. (*20*). It is not clear that additional hydroxyl groups on the B ring of these flavonols affect their NO inhibition activities as shown for their antioxidant activities (*22*). However, glycosylation of flavonols significantly decreased their inhibitory effects (**Table 1** and **Figure 2**); thus, the inhibitory effect of quercetin at 250 μM, for instance, was 63%, whereas those of quercetin 3-glucouside and quercetin 3-ruti-

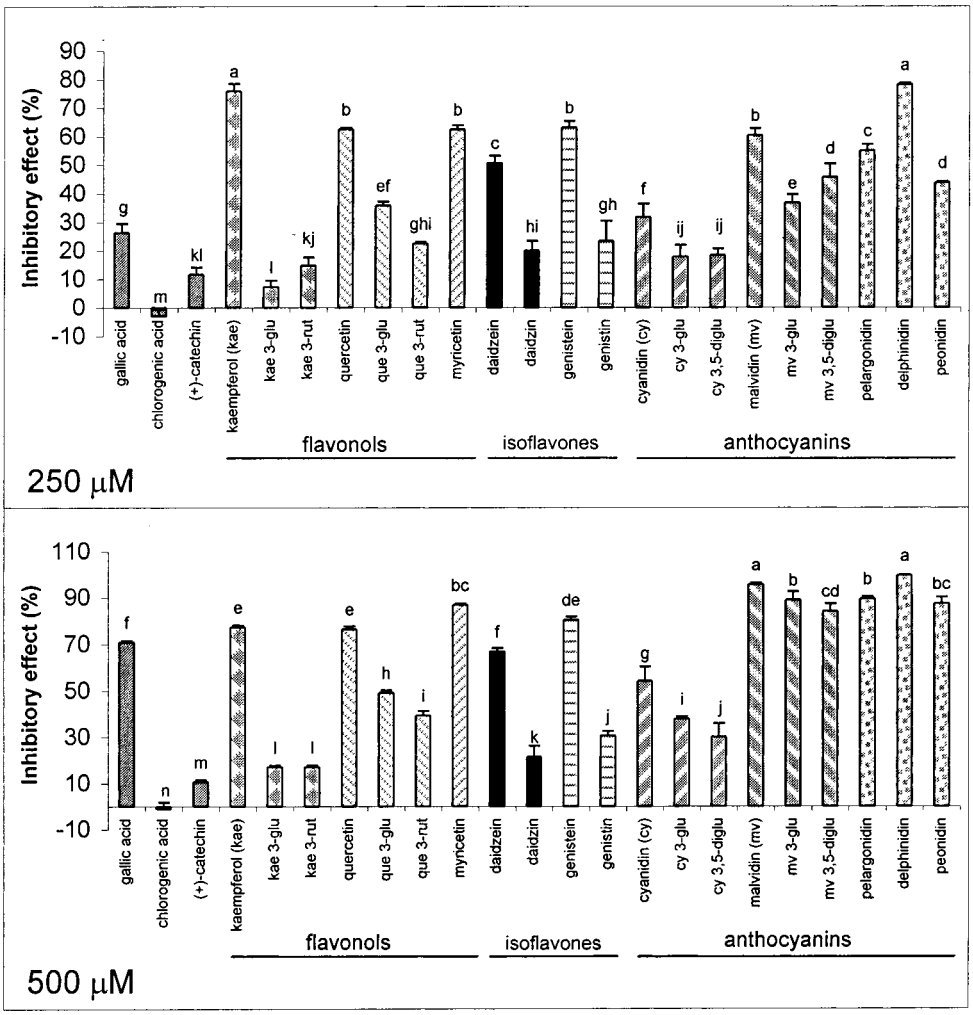

Figure 2. Inhibitory effects of phenolic compounds at 250 and 500 μM levels on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages. Each column represents the mean of triplicates. Error bars indicate standard deviations ($n = 3$). Columns with different letters denote significant difference in nitrite levels ($p < 0.05$).

noside (rutin) were 36 and 22%, respectively. The decreased inhibitory activity of glycosylated compounds has been attributed to the increased hydrophilicity and/or steric hindrance by additional sugar moieties, which may decrease absorption/penetration of glycosylated compounds into cells (*20*). There is also the possibility that the reduced inhibitory effects may reflect the lower free radical scavenging activity of glycosylated flavonoids (*22*). In addition, the glycosylated quercetin demonstrated a higher inhibitory effect than the glycosylated kaempferol (**Figure 2**), and the observed cytotoxicity of flavonol aglycons appears to decrease with increasing hydroxylation of the B ring (**Table 1**).

Isoflavones. The inhibitory effect of the isoflavones daidzein, genistein, daidzin, and genistin on NO production in LPS/IFN-γ-activated macrophages is illustrated in **Table 1**. Daidzin and genistin are the glycosylated forms of daidzein and genistein, respectively (**Figure 1**). The effects of isoflavones were dose-dependent. Daidzein and genistein, that is, aglycons, displayed cytotoxicity at high concentrations (250 or 500 μM) (**Table 1**). Daidzein exhibited moderate inhibitory activity and without significant cytotoxic effects. Under treatment levels of no significant cytoxicity, such as cell viability $\geq$ 60%, genistein and genistin had a stronger suppression effect than did daidzein and daidzin. Thus, a reduction of NO production by 22 and 36% was observed with genistein treatments of 63 and 125 μM, respectively, compared to 12 and 30% with daidzein at the same treatment levels. Glycosylation of isoflavones decreased their NO inhibitory activities (**Table 1** and **Figure 2**), and at treatment of 125 μM, NO production was decreased 36% by genistein but 7% by genistin. These inhibitory effects are in good agreement with previous results (*20*, *21*).

Anthocyanins. The inhibitory effect of anthocyanins on NO production and cell viability of LPS/IFN-γ-activated macrophages are shown in **Table 2**. All anthocyanins tested reduced NO content in cell supernatants in a dose-dependent manner and showed practically no toxicity toward cells. $IC_{\geq 50}$ values were 250 μM (IE = 55%) for pelargonidin, 500 μM (IE = 54%) for cyanidin, 250 μM (IE = 78%) for delphinidin, 500 μM (IE = 87%) for peonidin, 250 μM (IE = 60%) for malvidin, 500 μM (IE = 89%) for malvidin 3-glucoside, and 500 μM (IE = 84%) for malvidin 3,5-diglucosides. Anthocyanidins, that is, aglycons, had activities comparable to those of the aglycons of flavonols and isoflavones (**Figure 2**), yet the flavonol quercetin was more active at low concentration than the anthocyanins. Cyanidin and its derivatives showed less inhibitory effect than the other anthocyanins, and this may reflect a difference in the number of hydroxyl groups and/or degree of methylation of their hydroxyl groups in the five anthocyanidins examined. Glycosylation of anthocyanidins, like glycosylation of flavonols and isoflavones, reduced the inhibitory effect on NO production (**Table 2** and **Figure 2**). In the case of malvidin 3-glucoside and 3,5-diglucoside, however, the inhibitory effects on NO

Table 2. Effects of Anthocyanins on Nitric Oxide Production and Viability of LPS/IFN-γ-Activated RAW 264.7 Macrophages

concn (μM)	compound	nitrite[a] (μM)	cell viability[b] (%)	compound	nitrite (μM)	cell viability (%)	compound	nitrite (μM)	cell viability (%)
control[c]	cyanidin	2.0^{g} (0.1)		cyanidin	2.1^{f} (0.2)		cyanidin	2.1^{e} (0.2)	
LPS/IFN-γ[d]		48.5^{a} (1.2)	100 (0)	3-glucoside	52.7^{a} (2.1)	100 (0)	3,5-diglucosides	52.7^{a} (2.1)	100 (0)
16[e]		47.9^{ab} (2.7)	105 (2)		53.8^{a} (0.9)	111 (1)		53.2^{a} (1.1)	112 (1)
31		44.5^{bc} (1.3)	107 (2)		53.3^{a} (0.5)	110 (4)		51.2^{ab} (1.2)	107 (1)
63		43.5^{c} (0.2)	111 (2)		50.5^{b} (0.7)	111 (2)		50.0^{ab} (2.4)	108 (1)
125		39.6^{d} (2.4)	117 (2)		46.1^{c} (1.4)	107 (2)		48.1^{b} (2.2)	104 (1)
250		33.1^{e} (2.3)	127 (1)		43.3^{d} (2.1)	112 (2)		43.0^{c} (1.2)	109 (2)
500		22.3^{f} (3.1)	122 (3)		32.8^{e} (0.6)	113 (2)		36.8^{d} (3.1)	110 (3)
control	malvidin	1.4^{g} (0.1)		malvidin	1.4^{f} (0.1)		malvidin	1.4^{g} (0.1)	
LPS/IFN-γ		56.3^{a} (1.8)	100 (0)	3-glucoside	56.3^{a} (1.8)	100 (0)	3,5-diglucosides	56.5^{a} (2.3)	100 (0)
16		52.6^{b} (0.9)	114 (2)		57.1^{a} (2.6)	117 (3)		58.4^{a} (1.1)	107 (2)
31		50.1^{c} (0.8)	115 (2)		53.2^{b} (0.3)	110 (2)		53.3^{b} (0.9)	105 (2)
63		48.1^{d} (1.9)	117 (0)		50.8^{b} (2.5)	112 (3)		49.5^{c} (1.3)	102 (3)
125		39.2^{e} (1.2)	117 (1)		44.4^{c} (1.2)	112 (2)		45.3^{d} (1.1)	103 (2)
250		22.2^{f} (1.3)	116 (1)		35.6^{d} (1.6)	118 (0)		30.6^{e} (2.6)	107 (1)
500		24.^{g} (0.4)	71 (2)		6.1^{e} (2.0)	95 (8)		8.9^{f} (1.9)	93 (3)
control	pelargonidin	2.0^{g} (0.1)		delphinidin	2.0^{e} (0.1)		peonidin	1.4^{g} (0.1)	
LPS/IFN-γ		38.4^{a} (0.8)	100 (0)		38.4^{a} (0.8)	100 (0)		56.5^{a} (2.3)	100 (0)
16		38.5^{a} (0.3)	100 (2)		37.4^{a} (0.6)	106 (1)		52.7^{b} (2.4)	106 (3)
31		35.4^{b} (0.8)	99 (3)		34.6^{b} (1.1)	108 (1)		52.1^{b} (1.1)	107 (2)
63		29.7^{c} (1.1)	96 (4)		34.6^{b} (1.1)	108 (1)		52.1^{b} (1.1)	107 (2)
125		25.0^{d} (1.8)	97 (2)		25.0^{c} (0.8)	113 (0)		39.8^{d} (2.4)	108 (2)
250		17.3^{e} (0.8)	97 (6)		8.4^{d} (0.2)	111 (4)		31.7^{e} (0.3)	115 (0)
500		4.0^{f} (0.3)	111 (5)		0.1^{f} (0.0)	67 (2)		7.1^{f} (1.6)	113 (1)

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). Data for individual compounds with different letters denote significant difference in nitrite contents ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

Table 3. Phenolic Content[a] (Micrograms per Milligram, Freeze-Dried Material) of Berry Extracts and Their Concentrates

	fruit	total phenolics	tartaric esters	flavonols	anthocyanins
crude extracts	Saskatoon berry	26.4 (0.006)	4.2 (0.001)	3.5 (0.001)	8.7 (0.001)
	blueberry	33.5 (0.004)	3.7 (0.001)	3.4 (0.000)	14.3 (0.001)
	blackberry	75.7 (0.006)	3.5 (0.000)	2.5 (0.000)	16.5 (0.001)
	black currant	43.5 (0.003)	3.0 (0.000)	2.9 (0.000)	18.0 (0.000)
concentrates	Saskatoon berry	610.4 (0.036)	115.2 (0.006)	92.4 (0.005)	174.2 (0.017)
	blueberry	851.4 (0.046)	125.0 (0.016)	107.8 (0.014)	327.8 (0.025)
	blackberry	466.8 (0.015)	27.5 (0.003)	32.0 (0.002)	180.5 (0.002)
	black currant	539.4 (0.008)	46.1 (0.001)	42.9 (0.000)	228.3 (0.007)

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates ($n = 3$).

production were significantly larger than those of other glycosylated flavonoids and comparable to those of aglycons (**Table 2** and **Figure 2**). This may reflect a higher absorption of anthocyanins by the cells and/or a higher free radical scavenging activity of these compounds.

Berry Extracts and Concentrates. The berry crude extracts and concentrates contained high levels of phenolic compounds (2.6−85%) and anthocyanins (0.9−33%). It was expected that those phenolic compounds would demonstrate inhibitory activity on NO production in LPS/IFN-γ-activated macrophages. Berries were first extracted with 80% ethanol in water. After the removal of ethanol, aqueous extracts were freeze-dried. The freeze-dried samples, namely, crude extracts, were analyzed for their phenolic content (**Table 3**) and used for the determination of the effect on NO production. The inhibition on NO production by berry crude extracts and their concentrates is shown in **Table 4**. The crude extracts exhibited little effect on NO production, and this can be attributed to the low concentration of phenolics in these extracts (**Table 3**). At high concentration, 500 μg/mL, crude extracts of blackberries and black currants were moderately effective inhibitors of NO production. Extracts of Saskatoon berries and blueberries, however, had no effect on NO production.

When the crude extracts of berries were further purified as previously described, their relative phenolic content increased severalfold (**Table 3**). The total phenolic content of Saskatoon berries, blueberries, blackberries, and black currants increased 23-, 25-, 6-, and 12-fold from their crude extracts to concentrates; thus, the concentrates demonstrated considerable inhibitory effects on NO production compared to the crude extracts. Moreover, the correlations between phenolic content (at 250 and 500 μg/mL levels) and their inhibitory effects were pooled, and results are shown in **Figure 3**. The results clearly indicated that the inhibitory effects were significantly correlated with the content of individual categories of phenolic compounds present and expressed as total phenolics, tartaric esters, flavonols, and anthocyanins ($R^2 = 0.62-0.90$; $p < 0.01-0.05$). The results also show that the total phenolics and anthocyanins may be more associated with the NO inhibition ($R^2 = 0.76-0.90$; $p < 0.01$) than the tartaric acids and flavonols. As indicated in **Figure 2**

Table 4. Effects of Berry Crude Extracts and Concentrates on Nitric Oxide Production and Viability of LPS/IFN-γ-Activated RAW 264.7 Macrophages

	crude extracts		concentrates	
concn (μg/mL)	nitrite[a] (μM)	cell viability[b] (%)	nitrite (μM)	cell viability (%)
		Saskatoon Berry		
control[c]	1.2[c] (0.1)		1.6[e] (0.1)	
LPS/IFN-γ[d]	53.1[b] (2.2)	100 (0)	62.3[ab] (3.5)	100 (0)
16[e]	56.3[a] (0.7)	105 (1)	62.9[a] (2.8)	112 (1)
31	55.6[a] (1.3)	104 (2)	60.5[ab] (2.5)	111 (2)
63	55.9[a] (1.3)	104 (1)	58.3[bc] (1.0)	117 (0)
125	55.2[ab] (0.3)	106 (2)	58.5[bc] (2.1)	123 (1)
250	55.2[ab] (1.2)	108 (2)	54.8[c] (2.1)	127 (1)
500	54.8[ab] (1.5)	112 (2)	43.4[d] (2.8)	128 (0)
		Blueberry		
control	1.2[c] (0.1)		1.6[e] (0.1)	
LPS/IFN-γ	53.1[ab] (2.2)	100 (0)	62.3[ab] (3.5)	100 (0)
16	54.4[ab] (1.6)	101 (2)	61.2[a] (0.2)	112 (2)
31	56.5[a] (0.8)	99 (3)	61.5[a] (0.9)	113 (3)
63	55.0[ab] (0.5)	101 (4)	57.7[b] (2.7)	114 (1)
125	53.1[ab] (1.9)	100 (2)	48.8[c] (2.8)	119 (3)
250	51.8[b] (1.9)	103 (3)	21.2[d] (0.3)	123 (1)
500	51.6[b] (4.0)	109 (3)	2.6[e] (0.2)	111 (2)
		Blackberry		
control	1.9[f] (0.1)		1.8[f] (0.1)	
LPS/IFN-γ	55.1[bcd] (0.7)	100 (0)	62.2[ab] (2.7)	100 (0)
16	59.6[a] (3.2)	104 (1)	65.1[a] (2.9)	114 (6)
31	56.6[bc] (2.4)	105 (2)	62.5[ab] (0.8)	111 (3)
63	56.9[ab] (1.1)	105 (1)	60.2[bc] (0.6)	111 (1)
125	53.0[d] (1.0)	108 (1)	58.4[c] (2.7)	122 (2)
250	54.1[cd] (0.9)	111 (1)	52.5[d] (1.4)	129 (1)
	47.5[e] (1.4)	113 (1)	45.0[e] (2.2)	133 (0)
		Black Currant		
control	1.9[f] (0.1)		1.8[f] (0.1)	
LPS/IFN-γ	55.1[c] (0.7)	100 (0)	62.2[a] (2.7)	100 (0)
16	58.4[a] (1.8)	101 (1)	63.1[a] (1.3)	103 (5)
31	57.2[ab] (1.8)	100 (2)	60.8[ab] (0.7)	104 (2)
63	57.1[ab] (0.7)	103 (1)	59.0[bc] (1.3)	113 (5)
125	56.7[abc] (1.3)	106 (1)	56.8[c] (0.7)	113 (1)
250	55.9[bc] (0.9)	107 (3)	46.9[d] (2.9)	123 (2)
	52.3[d] (1.0)	113 (3)	24.2[e] (2.5)	132 (1)

[a] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates (n = 3). Data for individual extracts and concentrates with different letters denote significant difference in nitrite concenis ($p < 0.05$). [b] All data are means of triplicates, and numbers in parentheses indicate the standard deviation of these triplicates (n = 3). [c] Cells were not exposed to both LPS/IFN-γ and test compounds. [d] Cells were activated by LPS/IFN-γ but were not exposed to test compounds. [e] Cells were activated by LPS/IFN-γ and were exposed to test compounds.

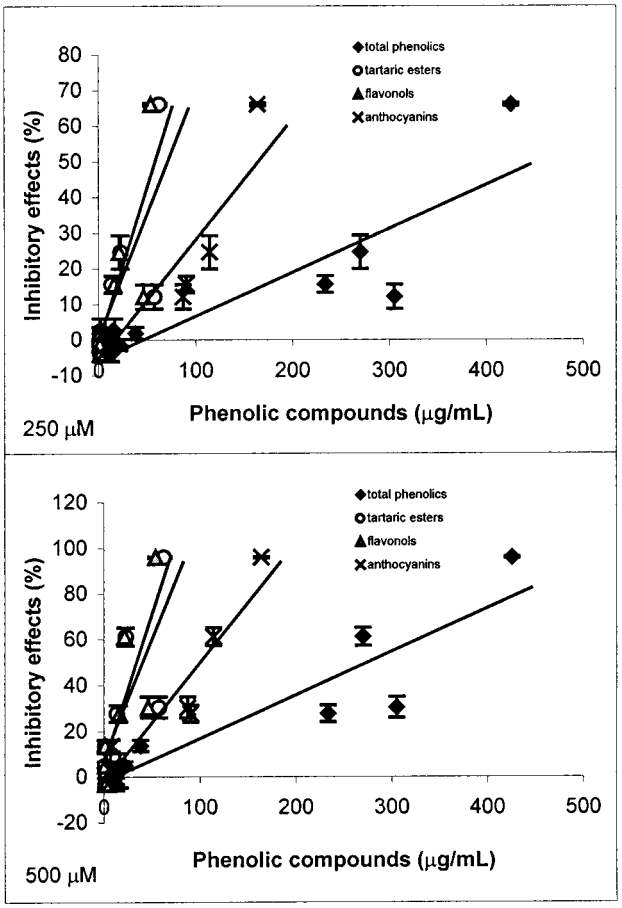

Figure 3. Correlations between the concentrations of phenolic compounds, from berry extracts and their concentrates, and their inhibitory effects on NO production of LPS/IFN-γ-activated RAW 264.7 macrophages. For treatment at the 250 μM level, the correlations are as follows: $y = 0.12x - 5.65$, $R^2 = 0.76$, $p < 0.01$, for total phenolics; $y = 0.70x + 0.38$, $R^2 = 0.61$, $p < 0.05$, for tartaric esters; $y = 0.87x - 1.05$, $R^2 = 0.66$, $p < 0.05$, for flavonols; $y = 0.34x - 5.86$, $R^2 = 0.83$, $p < 0.01$, for anthocyanins. For treatment at the 500 μM level, the correlations are as follows: $y = 0.19x - 2.2$, $R^2 = 0.83$, $p < 0.01$, for total phenolics; $y = 1.04x + 7.82$, $R^2 = 0.62$, $p < 0.05$, for tartaric esters; $y = 1.31x + 5.71$, $R^2 = 0.67$, $p < 0.05$, for flavonols; $y = 0.52x - 2.49$, $R^2 = 0.90$, $p < 0.01$, for anthocyanins. There are eight data points in each regression. Each data point represents the mean of triplicates. Error bars indicate standard deviations ($n = 3$).

and discussed above, individual anthocyanins may produce different inhibitory effects. Saskatoon berries and blackberries contain mainly glycosylated cyanidin (*28*, *29*); black currants contain glycosides of cyanidin, delphinidin, and pelargonidin (*30*); and blueberries contain glycosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (*30*, *31*). Thus, the higher inhibitory activity exhibited by blueberries may reflect differences in anthocyanins and other phenolics composition between berry extracts. However, more research on the effect of qualitative and quantitative composition of plant extracts on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages is required.

In conclusion, most phenolic compounds examined in this study showed inhibitory effects on NO production in LPS/IFN-γ-activated RAW 264.7 macrophages. Compounds that demonstrated >50% inhibition on NO production without showing cytotoxicity were the flavonols quercetin and myricetin, the isoflavone daidzein, and the anthocyanins/anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides. Anthocyanins were a very active group of flavonoids with high inhibitory effects on NO production. Their inhibitory effects were comparable to that of quercetin, which has been extensively studied and shown to exert anti-inflammatory and antioxidant effects. Anthocyanin-rich berry extracts also showed considerable inhibitory effects on NO production, and their inhibitory effects were significantly correlated with the content of total phenolics, tartaric ester, flavonols, and anthocyanins. Thus, consumption of a diet rich in fruits and vegetables may reduce the oxidative stress of nitric oxide and increase the protective effects against cardiovascular and chronic inflammatory diseases.

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