ANTHOCYANIN-RICH PHENOLIC EXTRACTS OF BLACK CHOKEBERRY (*ARONIA MELANOCARPA*) ATTENUATE INFLAMMATION INDUCED BY LIPOPOLYSACCHARIDE IN RAW 264.7 CELLS

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Abstract. *Aronia melanocarpa* berries and their extracts, have become well known for their notable health benefits. The present study investigated the anti-inflammatory effect of anthocyanin-rich phenolic extracts of Ame (*Aronia melanocarpa*) in LPS (lipopolysaccharide)-stimulated RAW 264.7 murine macrophage cells. The results showed that Ame pre-treatment significantly ameliorated oxidative stress and inflammatory biomarker activities, as evidenced by reductions in the production of ROS (reactive oxygen specie), MDA (malondialdehyde), and NO (nitric oxide), as well as suppression of iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase) and PGE2 (prostaglandin E2) mRNA levels; remarkably elevated the level of anti-inflammatory cytokine IL (interleukin)-10; and reduced the levels of the pro-inflammatory cytokines IL-1p, IL-6, and TNF-a (tumour necrosis factor). Additionally, we observed an attenuation of the cell apoptosis levels and the mRNA expression of apoptosis factors such as caspase-3 and caspase-9. In summary, the results highlight the health benefit of Ame against inflammation in LPS-stimulated RAW 264.7 cells.
Keywords: *Aronia melanocarpa anthocyanins, anti-inflammatory activity, cell apoptosis, apoptosis factors*

Introduction

Our inclusion of berries in the diet is gaining popularity due to their richness in health-beneficial nutrients, such as phenolic compounds, flavonoids, anthocyanidins and antioxidant vitamins (Hwang et al., 2014b). For the past several years, a growing amount of evidence has indicated that the consumption of plant foods rich in polyphenolic compounds is correlated with a lower risk of the development for oxidative stress-related diseases and has a beneficial effect beyond the actions of vitamins (Denev et al., 2012).

Recent studies have shown an increasing interest in *Aronia melanocarpa* (black chokeberry), which belongs to the Rosaceae family and originates from North America (Jakobek et al., 2012). It is a rich source of phenolic compounds, particularly

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proanthocyanidins and anthocyanins. Many earlier studies have shown that the concentrations phenolic compounds is many times higher than those in apples, red raspberries, blackberries, sweet rowanberries, blackthorn, sweet cherry, sour cherry, blueberries, raspberries, etc. (Castañeda-Ovando et al., 2009; Pellati et al., 2004; Polat et al., 2017). Aronia berries are extensively used for the production of juices, preserves, jams, wines and food colorants (Simić et al., 2016), and a number of health benefits have been ascribed to their intake (Mcdougall et al., 2016). In vitro and in vivo studies have demonstrated that black chokeberry has a wide range of positive effects, such as inhibition of cancer cell proliferation (Tao et al., 2017), antimutagenic effects, neuroprotective effects (Lee et al., 2017) and antidiabetic capabilities (Ciocoiu et al., 2017). It also displays several health-promoting properties in relation to chronic diseases, especially gastroprotective, hepatoprotective, and cardioprotective effects, which are related to its ant-inflammatory properties (Jurikova et al., 2017). Thus, aronia berries have potential as functional food ingredients. Inflammation is a complex physiopathological phenomenon that is mediated by activated inflammatory cells of the immune system, including macrophages (Yoon et al., 2012). It may induce various chronic diseases including cancer, cardiovascular diseases, Alzheimer's disease, type II diabetes, arthritis, metabolic syndrome, neurological diseases, and infectious diseases (Ahn et al., 2015; Hwang et al., 2014a). Lipopolysaccharide (LPS) is an endotoxin that is a potent inducer of inflammation and triggers the activation of macrophages that later release biomarkers of oxidative stress and inflammatory mediators, which then induce apoptosis (Khan et al., 2016).

Oxidative stress is considered a harmful disequilibrium between the generation and removal of radicals including lipid peroxidation products and ROS (reactive oxygen species) (Sivasinprasasn et al., 2016). Furthermore, ROS may damage biological molecules such as lipids, proteins and DNA and are crucial promoters of inflammation and cardiovascular disease (Rop et al., 2010). Inflammatory mediators include cell cytokines. Stimulated macrophages will release large amounts of cell cytokines such as IL (interleukin)-Ip, IL-6, IL-10, TNF-a (tumour necrosis factor) and other inflammatory mediators such as PGE2 (prostaglandin E2), iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase) during the inflammation process (Zdařilová et al., 2010). Thus, inhibition of the production of these inflammatory mediators is an important target in the treatment of inflammatory diseases. It has been reported that oxidative stress can impair function and trigger apoptosis (Isaak et al., 2017). Caspase-3 and Caspase-9 are directly involved in the process of apoptosis and are important pro-apoptotic molecules.

Compared with the synthetic anti-inflammation constituents, natural bioactive ingredients have higher efficiency and are economical. Besides, synthetic anti-inflammation constituents may exhibit toxicity and side-effects. Thus, it is necessary to search for more natural bioactive resources. Natural plant extracts including *Aronia melanocarpa* extract have shown beneficial effects on inflammation via the reduction of damage due to oxidative stress, apoptosis and modulation of inflammation cytokine expression.

Recently, works by Ah Ra Goh have indicated that *Aronia melanocarpa* extract exerts anti-inflammatory activities by inhibiting expression of pro-inflammatory mediators and ROS generation in HaCaT cells (Goh et al., 2016). Similar results were also obtained showing that intake of anthocyanin-rich black chokeberry juice can inhibit both the release of TNF-a, IL-6 and IL-8 in human peripheral monocytes and the activation of the NF-KB pathway in RAW 264.7 macrophage cells (Appel et al., 2015).

Herein, we investigated the anti-inflammatory effects and action mechanisms of *Aronia melanocarpa* extract and how it exerts an anti-inflammatory effect in LPS

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(lipopolysaccharide)-inflamed murine RAW264.7 macrophage cells in the present study. To the best of our knowledge, this is the first study that evaluated the anti-inflammatory properties of *Aronia melanocarpa* extract from three aspects: oxidative stress, inflammatory mediators, and apoptosis. The study further investigated whether *Aronia melanocarpa* extract could be used as a potential novel ingredient in anti-inflammatory health products or as a candidate drug for the prevention of inflammation.

Materials and methods

Materials and reagents

Ripe fruits of *Aronia melanocarpa* cultivars ("Fukangyuan Number 1") were harvested in Haicheng City (41°47'41"N, 122°40'42"E), Anshan Province, China. The *Aronia melanocarpa* were submitted to 60% alcoholic extraction, and the extracts were preserved at -20 °C for use. All the chemicals and reagents were purchased from Wanlei and Dingguo Biological Technology Co., Ltd. (Shenyang, Liaoning, China).

Phytochemical analysis

Anthocyanin phenolic acids

Anthocyanins were quantified by HPLC (High Performance Liquid Chromatography) experiments according to a previously described method (Wang et al., 2016). The column was a Dikma Platisil C18 column (4.6 mm × 250 mm inner diameter, 5 pm), and the solvent system used was 0.5% water solution in formic acid (A) and 100% HPLC grade acetonitrile (B) (elution conditions: 0-40 min from 0 to 40% B; 40-45 min, 40-45% B, 45-52 min, 0% B; flow rate 0.7 mL min^{-1}, injection volumes were 20 μL). Data were recorded at 520 nm. Anthocyanin components were quantified based on the calibration curves of structurally related external standards (cyanidin-3-glucoside). The standard concentration ranged from 0.5×10^{-3} to 1.5×10^{-3}mg/mL.

The phenolic acids were quantified using an HPLC system (Agilent 1100, Palo Alto, CA, USA) at 210 nm. They were separated using a 0.1% water solution of formic acid as solvent A and HPLC grade acetonitrile in 0.1% formic acid as solvent B (elution conditions: 0-45 min from 0 to 45% B; 45-52 min 0% B; flowrate = 0.7 ml min^{-1}; injection volumes 10 μ1). Each component was quantified based on the calibration curves of the structurally related external standards (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, benzoic acid, p-coumaric acid, ferulic acid, cinnamic acid). The standard concentration ranged from 2.5×10^{-5} to 50×10^{-5} mg/mL (Polat et al., 2017).

Total polyphenol content, anthocyanins, flavonoids and proanthocyanidin

The slightly modified method of Wang et al. (2016a) was applied to evaluate the total polyphenol content in the *Aronia melanocarpa* extracts. In short, 0.5 mL of the sample and 3 mL of Folin-Ciocalteu's reagent were incubated in the dark for 5 min at room temperature. Then, 2.4 mL of 7.5% sodium carbonate was injected and incubated for 2 h at room temperature in the dark. The absorbance values of the reaction mixture were then measured at 765 nm. Gallic acid (0-100 μg/mL) was used as the standard (mg GAE/g).

The total anthocyanin content in the samples was determined by the pH-differential method. Briefly, 0.025 M potassium chloride and 0.4 M sodium acetate were separately

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adjusted to pH 1.0 and pH 4.5 with hydrochloric acid. Next, 1 mL of the sample and 24 mL of buffer were incubated in the dark at room temperature for 15 min, and the absorbance was measured at 510 nm and 700 nm with distilled water as the blank control. Finally, the total anthocyanin content was calculated according to the formula given by Wang et al. (2016a).

The total flavonoid content of chokeberry cultivars was evaluated by the $NaNO_2$-AlNOs-NaOH method. The sample solution (0.1 ml) was mixed with 4 ml of ethanol (30%), followed by 0.5 mL of $NaNO_2$ (10%), 0.5 mL of $Al(NO3)_3$ (10%) and 4 ml of NaOH (4%). After incubation at room temperature for 30 min, the absorbance was measured at 510 nm and the total flavonoids content was calculated as rutin equivalents (mg RE/g).

The proanthocyanidin content of black chokeberries was evaluated by the method of Pedro et al. (2015) with modifications. Briefly, the sample solutions (1 mL) were mixed with 5 ml of 1% vanillin (1.0 g vanillin in 100 mL of methanol) and 10% conc-H_2SO_4 (10 mL conc-H_2SO_4 in 100 mL of methanol) at a proportion of 1:1 (v/v). After incubation for 30 min at 25 °C, the absorbance was measured at 500 nm and the proanthocyanidin content was calculated as catechin (mg CE/g).

Measurement of anti-inflammatory capacity

Cell culture and treatment

The mouse macrophage cell line Raw 264.7 (obtained from the cell bank of the Chinese Academy of Sciences, Shanghai, China) was cultured in complete DMEM (Dulbecco's Modified Eagle's Medium) with 10% heat-inactivated fetal bovine serum. Briefly, Raw 264.7 cells were seeded in 96-well and 6-well plates at a density of 10^3 cells/well and incubated at 37 °C, 5% CO_2 in a humidified incubator and allowed to attach overnight before the experiments. The Ame (*Aronia melanocarpa*) was filtered through a microfiltration membrane (0.22 μm) prior to addition to the culture media and was resuspended in DMEM to achieve a final concentration of 50 μg/mL. Cells were treated with the following:

i. DMEM only (control group)
ii. Ame added for 4 h daily for two consecutive days (Ame group)
iii. LPS at 1 μg/mL for 24 h (LPS group)
iv. Ame for 4 h daily for two consecutive days and then LPS at 1 μg/mL for another 24 h (LPS/Ame group)

The combination of dose/time for the Ame and LPS treatments was established based on preliminary MTT viability assays (data not shown). The cells and cell supernatants were collected and immediately frozen (-20 °C or -80 °C) until analysis.

Determination of ROS level

The measurement of ROS was performed according to the instructions given by the manufacturer of the kit (Shenyang Wanlei Bioengineering Institute, Shenyang, China). Briefly, DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate) was added to the serum-free culture medium to a final concentration of 10 μM. After incubation for 20 min at 37 °C, the cells were then collected and centrifuged at 1000 g for 10 min. Supernatants were removed carefully, after which the cells were resuspended in 200 μL of PBS and the fluorescence easily measured at $\lambda_{exication}$ 490 nm and $\lambda_{emission}$ 530 nm. ROS production levels for each treatment were normalized to the non-stimulated control and expressed as % control.

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Determination of lipid peroxidation

Lipid peroxidation was determined by measuring the MDA (malondialdehyde) in cells using a commercial MDA Kit (Shenyang Wanlei Bioengineering Institute, Shenyang, China). The absorbance was read on a microplate reader at 532 nm. MDA production levels for each treatment were normalized to the non-stimulated control and expressed as % control.

Nitric oxide (NO) inhibitory activity

The measurement of NO (nitric oxide) in Raw 264.7 cells was performed using a commercial NO kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The optical density was measured using a microplate reader at 550 nm. NO production levels for each treatment were normalized to the non-stimulated control and expressed as percent control.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a total RNA extraction kit, and 1 pg of RNA was used for cDNA synthesis using a Super M-MLV reverse transcriptase kit (BioTeke Corporation, Beijing, China) according to the manufacturer's protocol. After amplifying cDNA using real-time quantitative PCR with a SYBR green PCR Master Mix (Solarbio Life Sciences Institute, Beijing, China) according to our protocol, the levels of mRNA expression were quantified using an RT-PCR (RNA isolation and reverse transcription-polymerase chain reaction) system (ExicyclerTM 96, Bioneer, Daejeon, Korea). The primers' details used in this study are presented in *Table 1*. The thermal cycling parameters were as follows: 1 cycle at 94 °C for 5 min, 94 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s, followed by 40 cycles of 2.5 min at 7 °C, 1.5 min at 40 °C, melting for 34 s at 60 °C to 94 °C and 2 min at 25 °C. The nucleotide sequence of each primer and the size of the PCR products are shown in *Table 1*. mRNA expression was analyzed using the $2^{-\Delta\Delta C_T}$ method and normalized with respect to the expression of the p-actin housekeeping gene.

Apoptosis assay by flow cytometry

Annexin V-FITC/PI staining was performed to measure apoptosis by using an Apoptosis Detection Kit (Kaiji Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Briefly, cells from different treatment groups were collected and washed twice with cold PBS (phosphate buffered saline). The cell supernatant was carefully removed by centrifugation at 300×g for 5 min, and the cells were resuspended in 500 μL binding buffer, followed by the addition of 5 μL of Annexin V-FITC and 5 μL of PI (propidium iodide). After 15 min of incubation at room temperature in darkness, each sample was analyzed with flow cytometry (C 6, Becton Dickinson and Company, New Jersey, USA).

Statistical analysis

All experimental results are expressed as means and were performed in triplicate; the data in the tables and figures represent mean values ± standard deviation. One-way ANOVA (Analysis of variance) with Duncan's multiple range test was used to examine the differences between groups. Significant differences were considered to be $p < 0.05$ or $p < 0.01$.

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Table 1. *The oligonucleotide primer sets for the real-time PCR analysis*

Name	Sequence (5'—3')	Length	Tm	size
PGE2 F	GCCATTATGACCATCACCTTCG	22	61.8	250
PGE2 R	GCCATTATGACCATCACCTTCG	23	60.1	
iNOS F	GCAGGGAATCTTGGAGCGAGTTG	23	67.1	139
iNOS R	GTAGGTGAGGGCTTGGCTGAGTG	23	65	
COX-2 F	TTCCTCCCGTAGCAGATGACT	21	58.9	205
COX-2 R	AACCCAGGTCCTCGCTTA	18	55.1	
IL-βF	TGGTACATCAGCACCTCACA	20	54.7	132
IL-βR	GAAGGCATTAGAAACAGTCC	20	51.5	
IL-6 F	TGTATGAACAACGATGATGCAC	22	56.7	194
IL-6 R	CTGGCTTTGTCTTTCTTGTT	20	52.2	
IL-10 F	GAAGACAATAACTGCACCCACT	22	56.2	162
IL-10 R	ACCCAAGTAACCCTTAAAGTCC	22	56.5	
caspase-3 F	TGACTGGAAAGCCGAAAC	18	53.7	203
caspase-3 R	GGACTGGATGAACCACGAC	19	55	
caspase-9 F	CACTGCCTCATCATCAACAA	20	54.5	168
caspase-9 R	CATCAAAGCCGTGACCAT	18	54.1	
TNF-a F	AGAAAGCATGATCCGCGAC	19	58.3	236
TNF-a R	TTGTGAGTGTGAGGGTCTGG	20	55.8	
P-actin F	CTGTGCCCATCTACGAGGGCTAT	23	64.5	155
P-actin R	TTTGATGTCACGCACGATTTCC	22	63.2	

Results and discussion

Chemical composition

The detailed composition and contents of the *Aronia melanocarpa* extract are shown in *Table 2*. In the present study, 4 individual anthocyanins and 9 phenolic acids were identified in Ame. Ame possessed a high content of phenolic contents (655.11 ± 21.6 mg gallic acid equivalent/g), anthocyanins (195.76 ± 19.43 mg/g) and flavonoids (75.28 ± 6.82 mg rutin equivalent/g). HPLC-MS/MS (Liquid chromatography-mass spectrometry/mass spectrometry) analysis detected 4 anthocyanin pigments, with cyanidin 3-galactoside (92.44 ± 8.96 mg/g) and cyanidin 3-arabinoside (27.7 ± 4.51 mg/g) being the most representative anthocyanins in Ame. The present results indicated that anthocyanins predominated in the phenolic fractions of Ame, which are responsible for several beneficial actions in human health. That consistent with previous reports (Parzonko et al., 2015). The 9 main phenolic acids identified using HPLC methodology allowed the identification of Ame polyphenols based on standards. The most common and abundant phenolic acid compounds identified in Ame are benzoic acid and chlorogenic acid. These results are consistent with those previously reports (Polat et al., 2017).

The effects of Ame on biomarkers of oxidative stress: ROS and MDA

A sustained pro-inflammatory state, characterized by excessive ROS production, is the common denominator in the development, progression, and complication of many diseases (Gasparrini et al., 2017). For this reason, the measurement of ROS intracellular production could represent a very useful parameter to quantify oxidative stress induced by LPS. To investigate whether treatment with Ame influences LPS-induced ROS production, ROS was measured. According to our results, Ame itself caused no increase

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in basal ROS generation in RAW 264.7 macrophages ($p > 0.05$), while Ame significantly ($0.01 < p < 0.05$) suppressed the intracellular ROS production of LPS-stimulated RAW 264.7 macrophages based on 36% or higher changes relative to the LPS-stimulated controls (*Fig. 1A*). It suggesting that Ame phytochemicals may play a role in health maintenance by reducing oxidative stress (Goh et al., 2016).

Table 2. *Quantification and determination of total and individual phenolic compounds in Ame*

Compound[a]	Ame (mg/g)	Phenolic acid[c]	Ame (mg/g)
Total phenolic content	655.11 ± 21.6	Protocatechuic acid	0.102 ± 0.07
Flavonoid content	75.28 ± 6.82	P-hydroxybenzoic acid	0.055 ± 0.02
Proanthocyanidin content	0.06 ± 0.02	Chlorogenic acid	1.643 ± 0.31
Anthocyanins[b]		Caffeic acid	0.686 ± 0.17
Cyanidin 3-galactoside	92.44 ± 8.96	Benzoic acid	10.206 ± 1.22
Cyanidin 3-glucoside	4.04 ± 0.37	P-coumaric acid	0.295 ± 0.08
Cyanidin 3-arabinoside	27.7 ± 4.51	Ferulic acid	0.267 ± 0.10
Cyanidin 3-xyloside	6.21 ± 0.54	Cinnamic acid	0.253 ± 0.15
Total anthocyanins	195.76 ± 19.43	Gallic acid	0.045 ± 0.02

Values are means ± SD (n = 3)
[a]Individual phenolic compounds were compared with standard reference compounds
[b]Identified using HPLC-ESI-MS_2
[c]Identified using HPLC

Lipid peroxidation is a free-radical-mediated chain reaction involving several types of free radicals, which could be arrested through enzymatic means or by free radical scavenging by antioxidants and is considered one of the major manifestations of oxidative stress (Divya et al., 2015). Therefore, we used a lipid peroxidation assay to strengthen our findings in the above-mentioned regions. In the present study, we assessed the effect of topical administration of Ame during mouse macrophage cell LPS exposure by measuring the concentration of the short-chain aldehyde, MDA, which is the by-product of lipid peroxidation. As shown in *Figure 1B*, LPS application obviously enhanced MAD (machine analysis display). Pre-treatment with Ame significantly ($0.01 < p < 0.05$) reduced the MDA level, suggesting that Ame might be involved in the prevention of inflammation dysfunction via reducing the oxidative stress level in macrophages. These results obtained for the first time with *Aronia melanocarpa*, which are consistent with those previously reported by several authors, who tested the efficacy of different bioactive compounds against LPS-induced damage in macrophage cell models (Bak et al., 2013; Gasparrini et al., 2017; Lee et al., 2013).

The effects of Ame on inflammatory mediators

Inflammatory mediators: NO, PGE2, iNOS and COX-2

NO, PGE2, iNOS and COX-2 are the most important indicators for assessing inflammation injury. iNOS expression can increase the production of NO (Lee et al., 2013), which is also reported to affect the activity of COX-2 (Li and Wang, 2011). Similar to iNOS, COX-2 is also an inducible pro-inflammatory enzyme. COX-2 can convert arachidonic acid into PGE2, which can contribute to the pain and swelling associated with inflammation (Lee et al., 2013).

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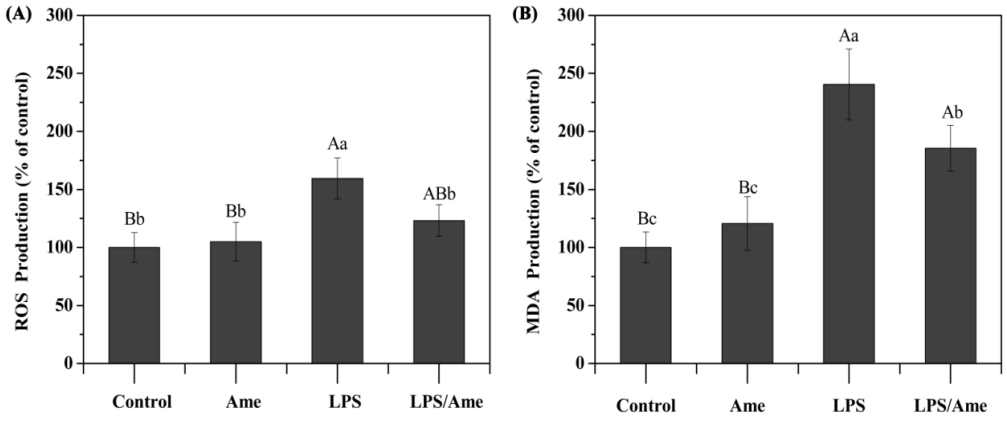

Figure 1. *The effects of Ame on biomarkers of oxidative stress (A and B): ROS and MDA. Ame, treatment with Ame alone; LPS, treatment with LPS alone; LPS/Ame, Ame pre-treatment followed by LPS treatment. The results are presented as the mean ± SD (n = 3). One-way (ANOVA) followed by Duncan's multiple range tests was performed to analyze the statistical differences among means. The different superscript lowercase letters denote significant differences between groups, and $p < 0.05$ or $p < 0.01$ was considered statistically significant*

The effects of Ame on the level of NO and PGE2 in the culture media of RAW 264.7 cells were determined after 24 h treatment with 1 μg/mL LPS. As shown in *Figure 2A*, there was no basal NO production during the incubation with only Ame without LPS ($p > 0.05$). After treatment with LPS, the NO concentration in the medium increased by approximately 2.45-fold (245.29%) compared to the control (100%). However, Ame at 50 μg/mL significantly inhibited the production of NO ($p < 0.01$).

The inhibitory effects of Ame on LPS-induced PGE2 secretion in RAW 264.7 cells were determined using RT-PCR (real-time polymerase chain reaction). As shown in *Figure 2B*, when the macrophages were not stimulated with LPS, PGE2 was almost undetectable in the medium with or without Ame ($p > 0.05$), while treatment with LPS caused an elevated production of PGE2 ($p < 0.01$), which was greatly reduced by Ame ($p < 0.01$). In this study, Ame effectively decreased NO production and PGE2 mRNA expression, indicating that Ame might be useful for suppressing the inflammatory process.

To investigate whether the Ame fractions had inhibitory activities against NO and PGE2 production via inhibition of iNOS and COX-2, RT-PCR analysis was used. As shown in *Figure 2C* and *D*, the expression of iNOS and COX-2 mRNA only showed an almost undetectable change between the unstimulated group and the Ame pre-treatment group. After LPS treatment, iNOS and COX-2 mRNA expression were markedly increased, whereas cotreatment with Ame significantly suppressed the expression of iNOS and COX-2 mRNA. These results are consistent with the inhibitory effect of Ame on NO and PGE2 release. The inhibitory profiles of Ame for iNOS and COX-2 overlapped with the profiles for NO and PGE2 production. On the basis of these results, it was concluded that Ame inhibited iNOS-mediated NO and COX-2-mediated PGE2 production. A similar finding was reported by a previous study, where blueberry extract was found to alleviate NO, PGE2 and COX-2 (Xu et al., 2016).

Inflammatory cytokines: IL-1β, IL-6, TNF-a and IL-10

Cytokines are a critical component of immune defense, but, on the other hand, inappropriate or excessive production of IL-1β, IL-6, TNF-a and IL-10 has been linked

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with the pathogenesis of a number of chronic inflammatory diseases (Yaqoob et al., 2010). IL-1β is known to induce fever and inflammation, finally leading to apoptosis. Moreover, it has been shown that IL-6 can be secreted by macrophages in response to specific microbial molecules to initiate the innate immune system (Yoon et al., 2009) and is a crucial checkpoint regulator of neutrophil trafficking by orchestrating chemokine production and leukocyte apoptosis (Fang et al., 2015). TNF-a is involved in many different cellular processes, including the production of numerous cytokines and acute phase proteins, and thus contributes to many pathophysiologic processes (Liu, 2005). On the other hand, IL-10 is a type of anti-inflammatory factor that down-regulates inflammatory responses and plays a role in inflammatory mediators of antagonism.

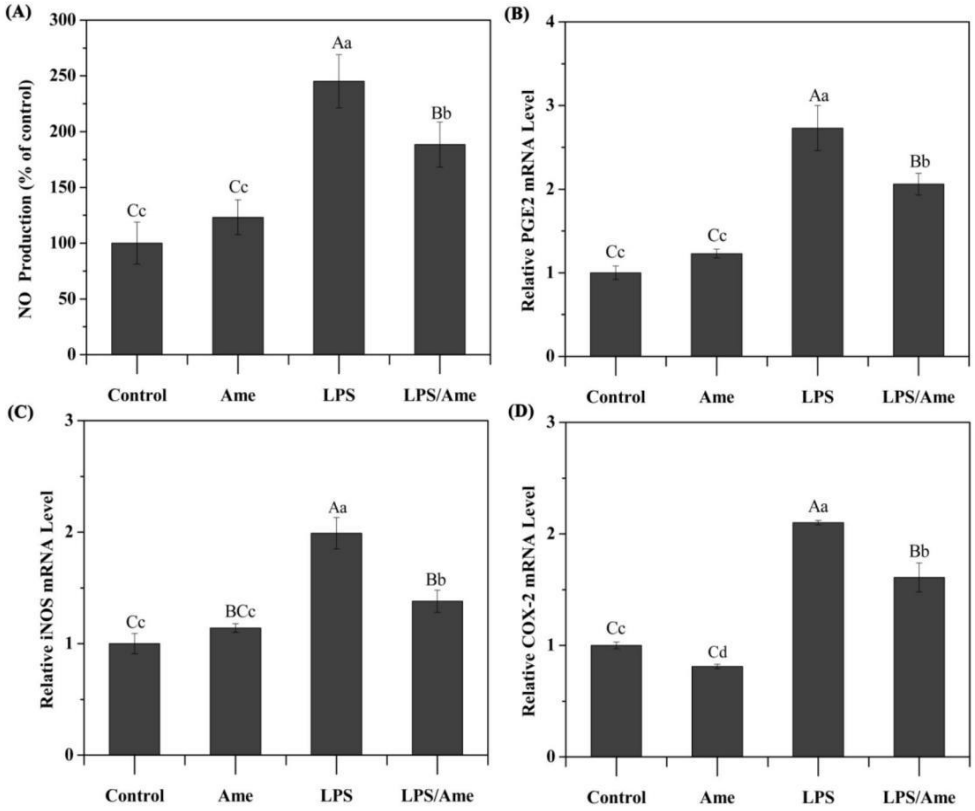

Figure 2. *The effects of Ame on inflammatory mediators (A, B, C and D): NO, PGE2, iNOS and COX-2. Ame, treatment with Ame alone; LPS, treatment with LPS alone; LPS/Ame, Ame pre-treatment followed by LPS treatment. The results are presented as the mean ± SD (n = 3). One-way (ANOVA) followed by Duncan's multiple range test was performed to analyze the statistical differences among means. The different superscript lowercase letters denote significant differences between groups, and $p < 0.05$ or $p < 0.01$ was considered statistically significant*

As shown in *Figure 3A, B* and *C,* Ame itself caused no increase in the aforementioned cytokine mRNA expression in RAW 264.7 macrophages ($p > 0.05$). When compared with cells treated with LPS alone, Ame resulted in an approximately 66%, 16% and 21% reduction in the mRNA expression of IL-1p, IL-6 and TNF-a, respectively. All of these reductions were significant ($p < 0.05$), especially IL-1β ($p < 0.01$). A different trend was found for IL-10: in unstimulated cells, IL-10 secretion increased to 104% ($p > 0.05$) of the control with 50 μg/mL Ame; in this case, an

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increase in IL-10 mRNA expression was observed with Ame + LPS treatments, with a significant increase ($p < 0.01$) compared to LPS-treated cells. The present results demonstrated that bioactive compounds in response to Ame exhibited a significant adjustment of inflammatory cytokines in RAW264.7 macrophages after exposure to LPS that helps decrease inflammatory damage.

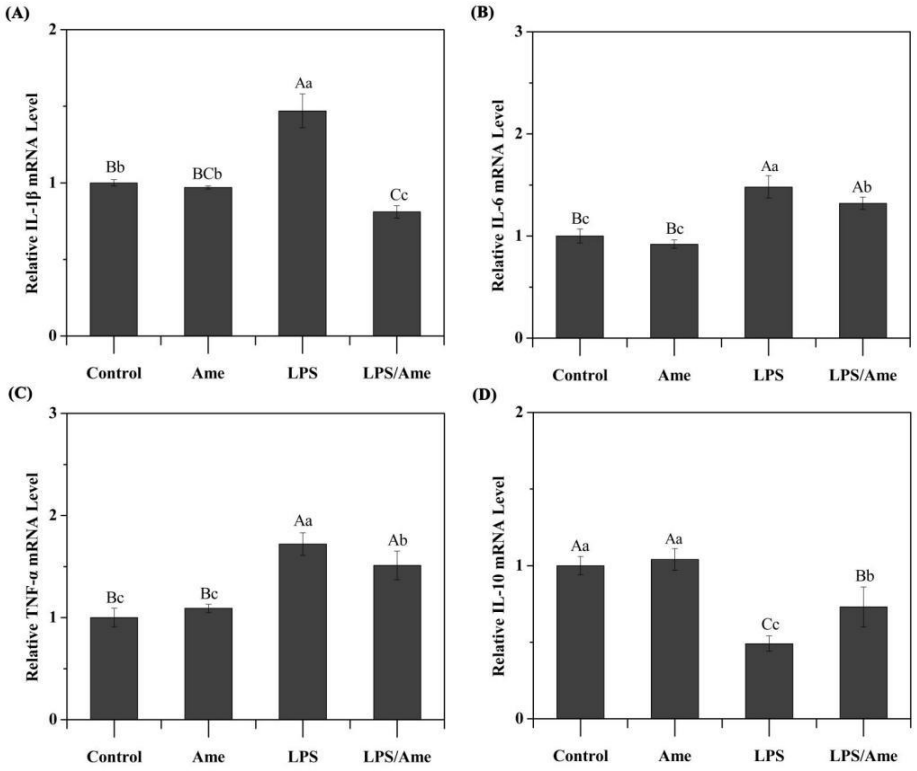

Figure 3. *The effects of Ame on inflammatory cytokines (A, B, C and D): IL-1p, IL-6, TNF-a and IL-10. Ame, treatment with Ame alone; LPS, treatment with LPS alone; LPS/Ame, Ame pre-treatment followed by LPS treatment. The results are presented as the mean ± SD (n = 3). One-way (ANOVA) followed by Duncan's multiple range tests was performed to analyze the statistical differences among means. The different superscript lowercase letters denote significant differences between groups, and p < 0.05 or p < 0.01 was considered statistically significant*

Previous reports (Appel et al., 2015) have suggested that the phenolics from chokeberry concentrate inhibit the release of TNF-a, IL-6 and IL-8 in LPS-induced RAW264.7 macrophages. These results are in line with the data obtained in other studies performed on RAW264.7 macrophages in which the expression of pro- and anti-inflammatory cytokines induced by LPS was improved by Ame (Appel et al., 2015) and other different bioactive compounds from strawberries (Gasparrini et al., 2017), blueberries (Wang et al., 2017), and *Lonicera caerulea* L (Wang et al., 2016b).

The effects of Ame on apoptosis

Biomarkers of apoptosis: caspase-3 and caspase-9

Caspases are known as important mediators of apoptosis and contribute to leading cells undergoing apoptosis to irreversible cell death. Caspase-3 and caspase-9 play major roles

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in the pathway of extrinsic apoptosis and endogenous apoptosis (Meng et al., 2017). Studies have shown that upregulation of caspase and TNF-a is an important factor in apoptosis (Chu et al., 2016). Therefore, we measured the activation of caspase-3 and caspase-9 with RT-PCR. As shown in *Figure 4A* and *B,* Ame itself caused no increase in caspase-3 and caspase-9 mRNA expression in RAW 264.7 macrophages ($p > 0.05$), while treatment with LPS for 24 h resulted in the activation of caspase-3 and caspase-9 as is apparent in comparison to the control groups ($p < 0.01$), but Ame treatment at 50 μg/mL significantly protected against caspase-3 and caspase-9 activation ($p < 0.01$). Interestingly, the mRNA expression of caspase-3 and caspase-9 after Ame + LPS treatment was similar to the control group ($p > 0.05$). These observations indicate that Ame can down-regulate mRNA expression of caspase-3 and caspase-9, thereby reducing macrophage cell death, which is an important step in preserving the immune system following inflammatory damage. In contrast to previous studies, we first evaluated the anti-inflammatory activity of Ame by exploring one aspect of apoptosis.

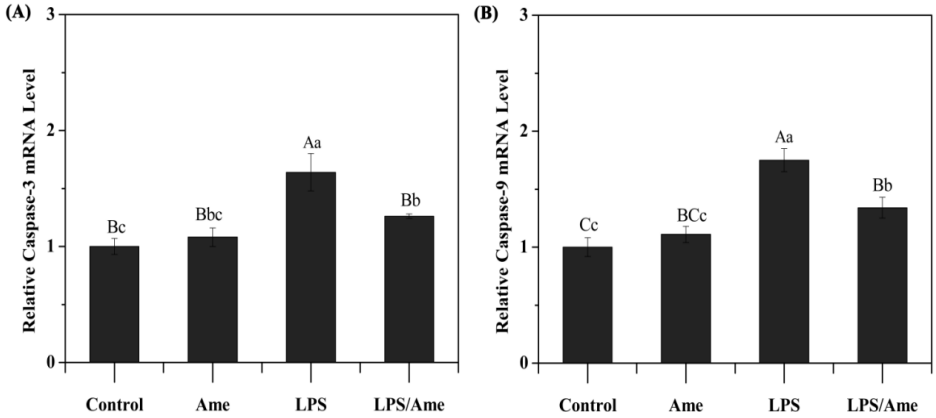

Figure 4. *The effects of Ame on biomarkers of apoptosis (A and B): caspase-3 and caspase-9. Ame, treatment with Ame alone; LPS, treatment with LPS alone; LPS/Ame, Ame pre-treatment followed by LPS treatment. The results are presented as the mean ± SD (n = 3). One-way (ANOVA) followed by Duncan's multiple range test was performed to analyze the statistical differences among means. The different superscript lowercase letters denote significant differences between groups, and p < 0.05 or p < 0.01 was considered statistically significant*

Apoptosis detected by flow cytometry

Flow cytometry was used to determine in which phase of the cell cycle the RAW 264.7 macrophages had accumulated and whether apoptotic events occurred in response to LPS exposure. As shown in *Figure 5*, in RAW 264.7 macrophages cells, LPS exposure groups resulted in typical apoptotic changes in the cells compared with cells from the untreated groups. When treated with 50 g/mL Ame, the apoptotic rate remarkably reduced the severity of apoptosis compared with the LPS groups (early apoptosis: 12.23% vs. 11.05%, late apoptosis: 21.65% vs. 11.01%, total apoptosis: 33.88% vs. 23.06%, respectively, $p < 0.05$). Treatment with Ame could therefore block RAW 264.7 cell apoptosis. Furthermore, Ame itself caused no increase in the apoptosis rate in RAW 264.7 macrophages ($p > 0.05$). The results showed that the early stages of apoptosis were lower than the late apoptosis. This also suggests that the reduction in cell inflammatory damage from the *Aronia melanocarpa* extracts was at least partially due to apoptosis of the RAW 264.7 cells.

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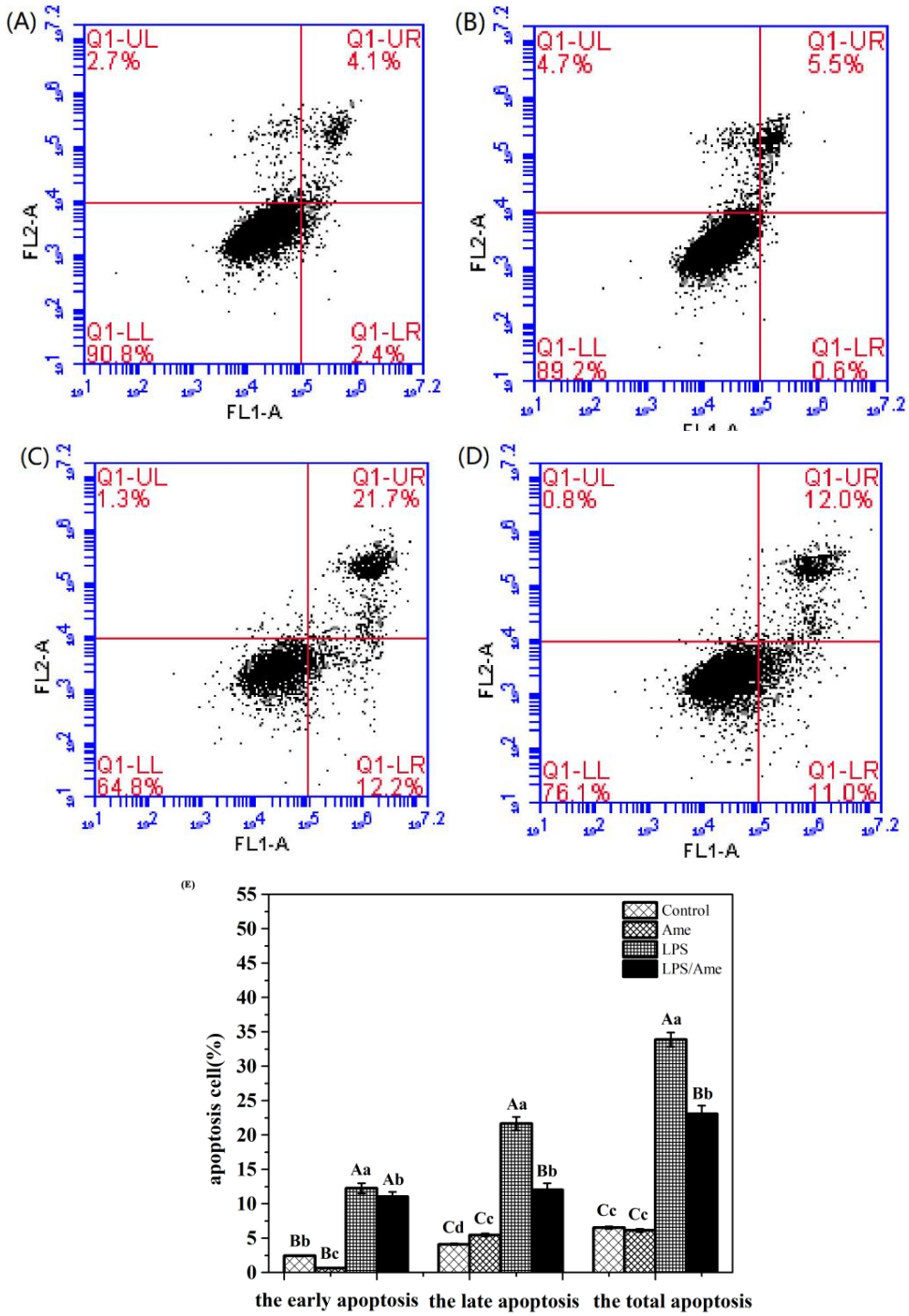

Figure 5. *The effects of Ame on apoptosis detected by flow cytometry. Images of flow cytometry detection of RAW 264.7 macrophage apoptosis (A, B, C and D). (A) Control; (B) Ame, treatment with Ame alone; (C) LPS, treatment with LPS alone; (D) LPS/Ame, Ame pre-treatment followed by LPS treatment. UL, necrotic cells. UR, late stage apoptotic cells. LR, early stage apoptotic cells. LL, live cells. (E) The results of flow cytometry detection of RAW 264.7 macrophage apoptosis in the early stage and late stage of apoptosis. The results are presented as the mean ± SD (n = 3). One-way (ANOVA) followed by Duncan's multiple range test was performed to analyze the statistical differences among means. The different superscript lowercase letters denote significant differences between groups, and $p < 0.05$ or $p < 0.01$ was considered statistically significant*

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Conclusions

In summary, we found that the *Aronia melanocarpa* extract, containing anthocyanins as its main phenolic components, could reduce LPS-induced inflammation by inhibiting the development of oxidative stress (via evaluation of ROS and MDA), regulating the activity of inflammatory mediators (NO, PGE2, iNOS and COX-2), attenuating the production of pro-inflammatory cytokines (IL-1β IL-6 and TNF-a), and increasing the expression of anti-inflammatory cytokines (IL-10). *Aronia melanocarpa* extract also attenuated the LPS-induced biomarkers of apoptosis: caspase-3 and caspase-9 prevented apoptosis of RAW 264.7 macrophages. Our results demonstrate that polyphenolic substances in *Aronia melanocarpa* extracts, especially anthocyanins, possess anti-inflammatory activities, *Aronia melanocarpa* extract has the potential to be developed as a novel ingredient in anti-inflammatory health products or as a candidate drug for the prevention of inflammation.

In this paper, *Aronia melanocarpa* were extracted and enriched to obtain freeze-dried powder with high anthocyanin content. The components in freeze-dried powder were identified by various methods, and its antioxidant ability was measured in vitro. Then its anti-inflammatory properties were explored from oxidative stress, inflammatory factors and apoptosis. On the basis of this paper, we can further supplement the paper and study the anthocyanin extract of Liriodendron nigra in more details and more perfectly.

The mechanism of anthocyanin prevention and protection of anti-inflammatory inhibition in *Aronia melanocarpa* needs further study. It can be deeply studied from the perspective of proteomics and combined with gene knockout technology to accurately find the gene or protein site regulated by anthocyanin.

The digestion, absorption and metabolism of anthocyanins in mice or human body need to be studied. Its metabolic transformation is studied by measuring metabolites in blood and urine.

This paper studies the inhibitory effect of anthocyanin extract of nigrum on inflammation by RT-PCR method β, tumor necrosis factor-α, iNOS, COX2, and reports that the above inflammatory mediators are regulated by NF-κB. We need to continue to study the next step.

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Conflict of interests. The authors declare that there is no conflict of interests.

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