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Anti-Inflammatory Effect of Berry Essences

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Anti-Inflammatory Effect of Berry Essences

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Abstract

Inflammation is one of defense reactions of our body tissues against harmful stimuli such as infection and tissue injury. However, it has been reported that chronic inflammation can lead to chronic inflammatory diseases including diabetes, cardiovascular diseases and cancer. This transition from acute to chronic inflammation state is usually induced by pro-inflammatory mediators and cytokines including nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which accelerate the development of other chronic diseases. Accordingly, targeting these pro-inflammatory factors is one of the important roles for anti-inflammatory agents. For last two decades, researches about anti-inflammatory effect of bioactive compounds from fruits and vegetables have increased. Among fruits and vegetables, berries are one of the most consumed fruits in our diet and have plenty of nutritive and non-nutritive components such as vitamins, minerals, and especially polyphenols and volatiles. Berry phenolic compounds are well known for having anti-inflammatory effect as well as antioxidant, anti-diabetic, and anti-obesity activities. However, berry volatiles, which are responsible for fruit flavor, have very limited information of health beneficial. Therefore, we investigated the anti-inflammatory effect of berry volatiles using LPS-stimulated RAW264.7 macrophage cell model, by studying the modulation of production of NO, IL-6, and TNF-α and comparing results to berry phenolics. We found that volatiles from six berries (blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry) have comparable anti-inflammatory activity to berry phenolics by reducing the production of NO, IL-6, and TNF-α *in vitro*. Results from this study suggest that berry volatiles may be potential effective treatments to prevent inflammation-related diseases.

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Introduction

Inflammation is one of our body's defense systems to fight infection, but chronic inflammatory conditions can result in various progressive diseases (Cheng *et al*., 2012). This process is very complicated and regulated by cytokines and pro-inflammatory factors, such as nitric oxide (NO), interleukin (IL)-6, IL-1ß, prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α) (Lee *et al*., 2016). If these factors are produced excessively due to prolonged inflammation, many diseases are more aggressive and acute. Thus, these cytokines and pro-inflammatory factors can be used as important targets for the development of anti-inflammatory agents (Soromou *et al*., 2012). Many researchers recently have studied bioactive compounds from fruits and vegetables, especially berries, which are polyphenol-rich fruits, to prevent inflammation. Berry phenolics were demonstrated to have significant beneficial health properties, including antioxidant, anti-diabetic, anti-obesity and anti-inflammatory activities (Qian *et al*., 2015). However, study of volatile compounds from berries, which are responsible for berry flavor and aroma, is very limited, compared to polyphenol compounds of berries. Thus, we **hypothesized** that berry volatiles have comparable anti-inflammatory effects to berry phenolics by modulating pro-inflammatory factors. Therefore, the **objective** of this study was to investigate the anti-inflammatory effect of berry volatiles using a RAW264.7 macrophage cell model, which is stimulated by lipopolysaccharide (LPS) to mimic the conditions of infection. The present study investigated whether volatile compounds from berries, like berry phenolics, modulate the production of pro-inflammatory mediator NO and cytokines (IL-6, TNF-α).

CHAPTER 1: Literature Review

1. Inflammation

Inflammation is the normal response in our body to fight infections, such as chemical stimuli and toxins which damage cells and tissues (Xie *et al*., 2012; Ham *et al*., 2015). The initiation of inflammation is tightly linked to the physiological homeostasis state (Ahmed, 2011). However, prolonged chronic inflammatory conditions can develop into human diseases, such as cancer (Huo *et al*., 2013; Dmitrieva *et al*., 2016), metabolic syndrome (Calder *et al*., 2011), diabetes (Agrawal and Kant, 2014) and cardiovascular diseases (Fearon and Fearon, 2008; Holvoet, 2008; Jang *et al.*, 2016). This pathogenic inflammatory process is regulated by cytokines and pro-inflammatory factors, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Lee *et al*., 2016).

1-1. Mechanism of Inflammation

Many chronic diseases, such as metabolic syndrome, diabetes, cardiovascular diseases and cancers, go through pathophysiological mechanism of inflammation (Holvoet, 2008). The mechanism of inflammation is a cascade which is regulated well. The innate immune hosting cells, such as macrophages and dendritic cells, recognize the pathogen or inflammatory stimuli by detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Ahmed, 2011; Libby, 2007). Damaged signals are recognized by Toll-like receptors (TLRs) and intracellular nucleotide binding domain and leucine-rich-repeat-containing receptors (NOD-like receptors, NLRs) (Chi *et al*., 2006; Xie *et al*., 2011; Ashley *et al*., 2012). TLR detects lipopolysaccharides (LPS), which is a strong activator of macrophages and a major component Gram-negative bacteria outer membrane, or endotoxin (Chi *et al*., 2006).

TLRs stimulate the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs), which are the pathway from extracellular to cellular response (Ashley *et al*., 2012). NF-κB, a transcription factor, regulates gene expression related to inflammation and is stimulated by pro-inflammatory cytokines. After activation by many cytokines or oxidative stress, IKK (IκB kinase) phosphorylates NF-κB-inhibitory IκBα protein and activated NF-κB can translocate into the nucleus and increase transcription of various pro-inflammatory cytokines which are soluble immune signaling molecules, including IL-1ß, IL-6, TNF-α, NO, and PGE_2 (Roger *et al*., 2001; Lee *et al*., 2016). Cytokines, chemokines and many inflammatory molecules increase the effector cells, including monocytes and neutrophils. Neutrophils induce toxic chemicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ashley *et al*., 2012). At the end of acute inflammation, the resolution state of inflammation is processed by anti-inflammatory mediators including IL-10, TGF-β and glucocorticoids. However, if the resolution of inflammation fails, the state of inflammation changes from acute to chronic (Ahmed, 2011). These mechanisms result in microbial metastasis and collateral damage to our body, including severe tissue damage and systemic inflammatory response syndrome (Lee *et al*., 2016).

1-2. Pro-Inflammatory Factors

The pathogenic inflammation is increased by regulation of pro-inflammatory mediators and cytokines, such as IL-1ß, IL-6, TNF-α, NO, and PGE_2 (Alvarez-Suarez *et al*., 2017). These pro-inflammatory factors are released excessively through transcription and translation of genes. IL-1β and IL-6 induce cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which produce PGE_2 and NO, respectively (Baud and Karin, 2001). COX-2 participates in many inflammatory processes and induces many carcinomas (Hugo *et al*., 2015). NO is a short-living

lipophilic free radical that controls homeostasis in many physiological and biological systems (Cheng *et al*., 2015). NO is synthesized by oxidizing L-arginine, using the nitric oxide synthases (NOS). There are three known isoforms of NOS: neuronal (nNOS) and endothelial nitric oxide synthases (eNOS) produced constitutively and iNOS produced by immune cells such as macrophages. nNOS and eNOS produce low levels of NO and regulate physiological and biological functions, but iNOS produces a high level of NO by activating NF-κB, with the stimulation of TNF-α, interferon-gamma (IFN-γ), IL-6, interleukin-1alpha (IL-1α), LPS, bacterial and viral component (Soufli *et al*., 2016). The production of NO is considered as an indirect indicator of inflammation because excessively produced NO produces superoxide anion (O_2^-), which can form peroxynitrite ($ONOO^-$) (Guzik *et al*., 2002). Peroxynitrite ($ONOO^-$) shows cytotoxic effects, including DNA damage, oxidation of LDL and other inflammatory diseases. IL-6 is a cytokine released by many cells, such as inflammatory cells, prostate cancer cells and prostate stromal cells (Guzik and Adamek-Guzik, 2003). It has a key role as an endogenous mediator of immune response to infections. IL-6 controls acute inflammation by regulating differentiation and activation of B-cells and T-cells, and supports cell growth and survival (Nguyen *et al.,* 2014). In addition, IL-6 causes change from acute inflammatory state to a more chronic pro-fibrosis state by increasing T helper 1 (Th1) cells (Fielding *et al*., 2014). TNF-α is also one of the most important pro-inflammatory mediators. It has a potential toxic effect, which results in hypersensitive responses with prolonged inflammation, and increases when some pathogenic conditions occur (Soromou *et al*., 2012). Accordingly, these cytokines and pro-inflammatory mediators have been used as important targets for the development of anti-inflammatory agents.

1-3. Inflammation and Health

A. Inflammation and Cancer

Chronic inflammation is one of the major factors of tumor development. According to an epidemiological study, 15 to 20% of many different cancer types in the world are related to chronic infections and inflammations (Dmitrieva *et al*., 2016). It has been postulated that chronic inflammation can promote cancer, especially tumorigenesis (Huo *et al*., 2013). A lot of different immune cells can be commonly found in tumors (Ahmed, 2011). Prolonged inflammatory microenvironment destabilizes immune responses, damages DNA and enhances tumor promotion, progression, and invasion of other tissues, angiogenesis, and metastasis (Lin and Karin, 2007). Nguyen *et al.* (2014) suggested that IL-6 has a key role in promoting most steps of pathogenesis of prostate cancer, including initiating prostate tumorigenesis, stimulating tumor growth, progressing prostate cancer, promoting tumor metastasis and increasing resistance to chemotherapy. IL-6 can increase prostate cancer cells and reduce apoptosis through many different signal pathways such as mitogen activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI3-K) pathway and signal transducer and activator of transcription (STAT) pathway (West *et al*., 2015). NF-κB has an important role in inflammation and tumor initiation. It regulates not only pro-inflammatory cytokines, but also tumor cell death, progression of tumor cell cycle, generation of new tumors and metastasis by regulation of related gene expression (Dobrovolskaia and Kozlov, 2005). IL-1β, another activator of NF-κB, increases in several cancer. Wang *et al*. (2014) reported that high level of IL-1β increased in tumor microenvironment via neutrophils in colitis-associated cancer mouse model. TNF-α also has been considered to initiate tumor progression by stimulating genotoxic molecules, leading to DNA damage and mutations (Hussain *et al*., 2003; Lin and Karin, 2007).

B. Inflammation and Metabolic Syndrome

Metabolic syndrome is a common, but complex disorder combining obesity, dyslipidemia, glucose intolerance, hypertension, and insulin resistance. These disorders are associated with various chronic diseases including type 2 diabetes, cardiovascular disease, osteoporosis, and several cancers (Holvoet, 2008; Lee *et al*., 2014). High energy and high fat diet can increase inflammatory stress (Calder *et al*., 2011). Cani *et al*. (2007) reported that continuously LPS-infused mice showed similar weight gain to high fat diet-fed mice, while LPS response-lacking mice were protected from high fat diet-induced obesity. Central obesity is induced by adipose-released cytokines (Ouchi *et al*., 2011). Abnormally enlarged adipocytes also increase and activate macrophages by increasing monocyte chemoattractant protein-1 (MCP-1). Increased macrophages promote pro-inflammatory state by releasing many pro-inflammatory proteins including TNF-α, IL-6, and MCP-1 (Joseph *et al*., 2016). In addition, ROS induced by excessive energy intake increases metabolic oxidative stress, which activates pro-inflammatory signaling pathways such as NF-κB, and c-Jun N-terminal kinase (JNK) (Calder *et al*., 2011). Dyslipidemia is an abnormally accumulated lipid in the vessel. Dyslipidemic patients showed increased plasma TNF-α levels and this suggested TNF-α as a marker for hyperlipidemia (Pauciullo *et al*., 2008). In hypertension, inflammation is involved in both direct and indirect mechanisms. In direct mechanism, C-reactive protein (CRP) elevates vasoconstriction and oxidation by inhibiting NO production. CRP in the adipose tissue and liver acts as an acute-phase protein (Parelman *et al*, 2012). Inflammation also promotes hypertension indirectly through insulin resistance which is increased by inflammation (Scheede-Bergdahl *et al*., 2009). Thus, many researchers suggested inclusion of pro-inflammatory state as part of the metabolic syndrome (Ikonomova, 2004).

C. Inflammation and Diabetes

Chronic inflammation is involved in the pathogenesis of type 2 diabetes mellitus (T2DM) (Shu *et al*., 2012). Inflammation decreases beta cell secretory capacity and increases insulin resistance and islet cell inflammation (Agrawal and Kant, 2014). Cytokines from macrophages, including IL-1ß and TNF-α, play an important role in initial islet ß-cell damage (Saxena and Modi, 2014). Cytokines, including TNF-α, IL-1ß, IL-1Ra, Il-6, IL-18, and IL-10, cause inflammation and immune responses, leading to diabetes (Banerjee and Saxena, 2014). TNF-α decreases activity of tyrosine kinase in insulin resistant rodents and humans, suggesting TNF-α as a mediator of insulin resistance and diabetes (Fontaine-Bisson *et al*., 2007). Studies of type 2 diabetic patients reported that CRP and IL-6, main stimulators of the production of acute phase proteins, elevated in these diabetic patients compared with nondiabetic controls (Wang *et al*., 2013).

D. Inflammation and Cardiovascular Disease

Inflammation is related to the development and progression of many cardiovascular conditions such as coronary artery disease, high blood pressure, stroke, atherosclerosis and congenital heart disease (Berg and Scherer, 2005; Fearon and Fearon, 2008). Atherosclerosis is one of cardiovascular diseases that usually begins early in life (Pockley, 2003). Inflammation especially plays an important role in the development of atherosclerosis after initiation. The most crucial early step at atherosclerotic sites is recruiting and activating macrophages which produce cytokines and inflammatory mediators, inducing cell migration, proliferation, extracellular matrix production, and plaque development (Tousoulis *et al*., 2016). Among these markers, cytokines, such as IL-6 and TNF-α, showed more specific inflammatory signaling at early state. These pro-inflammatory cytokines can be used as a biomarker for predicting cardiovascular

mortality (Myers *et al*., 2004). Rheumatoid arthritis can be characterized by chronic inflammation in various body systems, including the joints, eyes, lungs, heart and blood vessels. The connective tissue of the joint in rheumatoid arthritis, the synovium, activates macrophages, lymphocytes, and synovial cells by chronic inflammation. Activated neutrophils invade synovial fluid from synovial cells which increases chronic inflammatory state in synovial tissues (Ahmed, 2011).

1-4. Prevention of Inflammation

Targeting of inflammatory mediators or pro-inflammatory cytokines can induce anti-inflammatory effect and preventive effect of inflammation (Mantovani *et al*., 2008). In addition, many factors, including diet, lifestyle, and medication, can prevent or modify inflammatory processes and promote healthier life (Libby, 2006). Dietary components, especially natural compounds from food have been reported for many beneficial health effects, especially in prevention of inflammatory, infectious, and chronic diseases (Jayaprakasha *et al*., 2013). Bioactive compounds from fruits and vegetables showed a number of health benefits in prevention of inflammation and chronic diseases (Patil *et al*., 2009; Jayaprakasha *et al*., 2013).

2. Berries

Berries, one of the most common fruits in the human diet, are rich in natural compounds, such as minerals, vitamins, dietary fibers, and especially polyphenols and volatiles (Battino *et al*., 2009; Afrin *et al*., 2016). The most common berries consumed in the United States are blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberries. Less consumed are acai, black currant, chokeberry, and mulberries (Basu *et al*., 2010). It has been reported that berry polyphenols showed many different health beneficial effects (Qian *et al*., 2015; Wang *et al*., 2013; Lim *et al*., 2013). Wang *et al*. (2013) and Lim *et al*. (2013) reported that mulberry fruit

berry extracts, which contain many polyphenols, showed antioxidant (Skrovankova et al., 2015; Genskowsky *et al.*, 2016), anti-diabetic (Stote *et al.*, 2017), anti-obesity (Noratto *et al*., 2016) and anti-inflammatory activities (González-Gallego *et al.*, 2014; Chew *et al*., 2018). However, Nile *et al*. (2014) pointed out that there are very limited studies of the biological activities of volatile compounds from berries that are responsible for the flavor of berries.

2-1. Phytochemicals in Berries

A. Berry Polyphenols

Phenolic compounds are produced as secondary metabolites in plants (Martin and Bolling, 2015). The structure of phenolic compounds consists of benzene rings with single or multiple hydroxyl substituents (H Farzaei *et al*., 2015). They can be from simple phenolic molecules to highly polymerized compounds (Velderrain-Rodríguez *et al*., 2014). They are natural compounds from plants, such as fruits, vegetables, tea, coffee and wine. Phenolic compounds of berries contain flavonoids (flavonols, flavones, flavanols, and anthocyanins), tannins (proanthocyanidins and ellagitannins), phenolic acids (hydroxy-benzoic acids and hydroxy-cinnamic acids), and stilbenes (Figure 1) (Folmer *et al.,* 2014; Nile *et al*., 2014). Most well-studied compounds are anthocyanins, proanthocyanidins, and other flavonoids. Anthocyanins are the most abundant group in berry flavonoids (Johnson *et al*., 2013). Cyanidin, malvidin, pelargonidin, peonidin, and petunidin are the most common anthocyanidins in berries (Edirisinghe and Burton-Freeman, 2016). They are water-soluble and responsible for the pigments of berries, providing red to blue color and functioning physiologically in plants (Bueno *et al*., 2012). Proanthocyanidins give astringent tastes to berries. Among commonly consumed berries, cranberries and blueberries contain abundant amounts of proanthocyanidins (Seeram 2008, Nile *et al*., 2014). The total amount of polyphenol compounds (anthocyanidins,

proanthocyanidins, phenolics, flavonols, flavanols and ellagitannins) in five different berries (blackberry, blueberry, cranberry, raspberry and strawberry) is shown in Table 1. Total phenolic compounds in berries can vary among species, cultivars, and growing conditions of berries (Edirisinghe and Burton-Freeman, 2016).

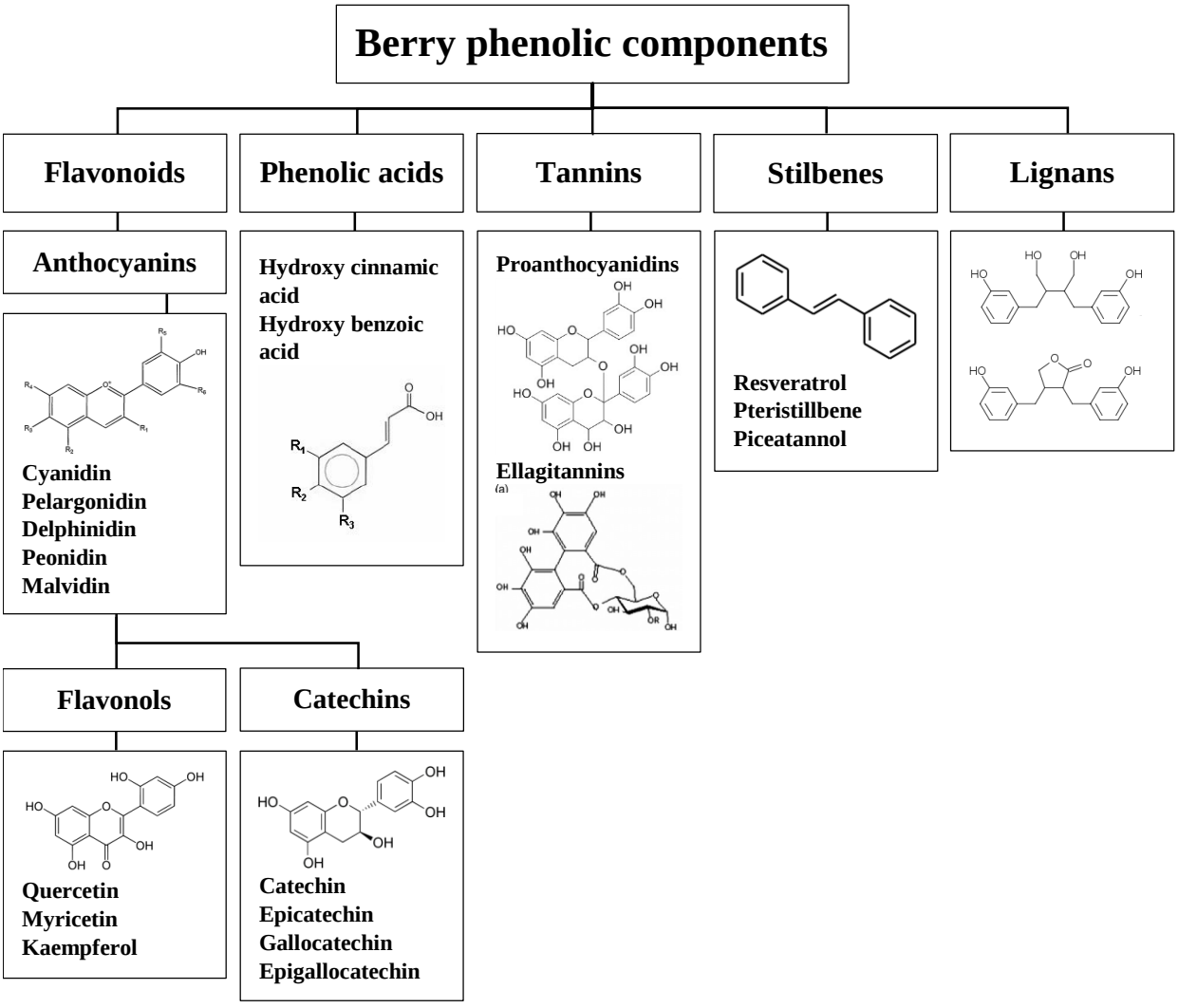

Figure 1. Phenolic Components in Berries (Modified from Nile *et al*., 2014)

Table 1. Polyphenol Compounds of Some Common Edible Berries (Adapted from Folmer *et al*., 2014)

	Anthocyanidins (mg/kg FW)	**Proanthocyanidins** (mg/kg FW)	**Phenolics** (mg/kg FW)	**Flavonols** (mg/kg FW)	**Flavanols** (mg/kg FW)	**Ellagitannins** (mg/kg FW)
Blackberry	900	270	650	N.a.	37	1,000
Blueberry	4,300	1,798	1,300	135	40	20
Cranberry	4,370	4,188	300	260	73	N.a.
Raspberry	3,240	302	N.a.	12	44	250
Strawberry	340	660	N.a.	600	42	200

FW = fresh weight; N.a. = not analyzed

B. Berry Volatiles

Aroma is one of the most significant characteristics that affects the flavor of a fruit, and its acceptability and consumption. In berries, volatile compounds are responsible for the flavor and aroma of berries and are not uniformly distributed (Dymerski *et al.,* 2015; Fenoll *et al.,* 2009). Dymerski *et al*. (2016) compared unextracted gooseberry chromatogram with the ones of cranberry and blueberry. They found volatile compounds in each cranberry and blueberry sample which have similar chemical families such as terpenes, esters, carboxylic acids, ketones, aldehydes and alcohols. The volatile compounds which are highest in each berry were different, which can affect their different biological activities in humans. Blueberry had the highest amount of alcohol and ester components, cranberry contained carboxylic acids, ketones and aldehydes most, and terpenes were predominant in cape gooseberry. Volatile compounds identified from blackberry and strawberry are shown in Table 2 (Jacques *et al.,* 2014) and Table 3 (Oz *et al*., 2016), respectively. Jacques *et al*. (2014) identified volatile compounds from blackberry by using solid-phase micro extraction (SPME). The most abundant compounds were terpenoids, and limonene was the major terpenoid in blackberry. Ethyl hexanoate, furaneol, geraniol, β-ionone, linalool and *trans*-2-hexenol are related to fresh fruit, floral, raspberry and strawberry aromas and eugenol, myrtenol, 1-octen-3-ol and α-terpineol are related to moldy, vegetal and woody flavors (Du *et al*., 2010). In Table 3, Oz *et al*. (2016) detected volatile compounds from four different strawberry varieties (Fortuna, Rubygem, Sweet Ann and Ventana) by using headspace solid-phase micro-extraction gas chromatography mass spectrometry (HS-SPME/GC-MS). The most detected compounds were esters (31 compounds), which give fruity and floral characteristics in strawberry (Dong *et al*., 2013). Among esters, hexanoic acid ethyl ester was predominant in strawberries. In Table 4, Lim *et al*. (2012) identified volatile compounds from

Korean black raspberry juice. Malowicki *et al*. (2008) identified volatile compounds in three different red raspberry fruit cultivars grown in Washington in 2005 (Table 5).

Table 2. Volatile Compounds Identified in Blackberry (Cultivar 'Tupy') (Modified from Jacques *et al*., 2014)

Class	Name	Area %	RT (min)	S
Terpenoid		**75.38**		
	α-thujene	0.18	4.65	91
	α-pinene	0.44	4.80	97
	camphene	0.24	5.11	97
	β-myrcene	1.16	5.91	95
	α-phellandrene	0.59	6.26	96
	terpinolene	0.60	6.56	96
	limonene	63.26	6.95	94
	α-terpinene	0.73	7.56	97
	linalool oxide	0.52	7.91	98
	o-cimene	0.96	8.32	91
	linalool	0.79	8.58	96
	trans limonene oxide	0.25	9.64	92
	isopinocarveol	0.24	9.72	93
	isoborneol	0.36	10.45	90
	terpinen-4-ol	1.12	10.73	91
	p-cymen-8-ol	1.13	10.96	93
	α-terpineol	0.78	11.11	95
	(-)-carvone	0.96	12.56	95
	geraniol	0.37	12.82	95
	vitispirane	0.39	13.57	95
	theaspirane	0.17	14.11	92
	α-copaene	0.13	16.19	93
Hydrocarbon		**4.35**		
	heptane	3.58	2.06	96
	toluene	0.77	2.57	94
Ketone		**4.23**		
	methyl ethyl ketone	3.65	1.68	95
	2-heptanone	0.17	3.98	98
	verbenone	0.23	11.64	92
	damascenone	0.18	16.40	92
Alcohol		**4.06**		
	2-heptanol	3.24	4.13	98
	1-heptanol	0.22	5.47	91
	1-octanol	0.60	7.83	92
Ester		**0.76**		
	methyl-hexanoate	0.17	4.56	92
	ethyl-hexanoate	0.47	6.07	98
	ethyl benzoate	0.30	10.53	94
	methyl salicylate	0.76	11.24	96
Aldehyde		**0.53**		
	hexanal	2.05	2.81	91
	2-hexenal	1.52	3.45	97
	heptanal	4.55	3.65	98
	heptenal	0.23	5.19	94
	benzaldehyde	0.30	5.36	90
	octanal	0.22	6.16	96
	nonanal	0.47	8.66	95
	nonenal	0.22	10.16	92
	decanal	0.19	11.40	95
	p-mentenal	0.53	11.77	95
Area total % identified			**77.14**	

MW = molecular weight; S = similarity with mass spectra; % area = area % related to the total peak area

Table 3. Volatile Profiles of Four Strawberry Fruit Varieties Detected by HS-SPME/GC-MS, % (Adapted from Oz *et al*., 2016)

Class	Compound	Cultivar			
		Fortuna	**Rubygem**	**Sweet Ann**	**Ventana**
Acid		**0.9**	**28**	**0.6**	**28**
	acetic	N.d.	5.72	1.12	7.41
	peracetic	3.41	3.41	N.d.	N.d.
	butanoic	N.d.	N.d.	N.d.	2.76
	2-methyl-3-hydropropanoic	N.d.	1.18	N.d.	N.d.
	1,2-benzenedicarboxylic	N.d.	N.d.	1.18	N.d.
Alcohol		**0.5**	**28**	**14**	**24**
	2-methyl-*z*,*z*-3,13-octadecadienol	4.11	N.d.	0.62	N.d.
	2*H*-pyran-3-ol	N.d.	N.d.	1.89	N.d.
	ethanol	1.74	N.d.	N.d.	14.03
	3-amino-1-propanol	N.d.	N.d.	8.17	N.d.
	1,6-octadien-3-ol	N.d.	N.d.	1.89	N.d.
	1-nonanol	N.d.	0.43	N.d.	N.d.
	β-fenchyl	N.d.	N.d.	2.92	0.63
	2-methyl-4-hexen-3-ol	N.d.	N.d.	N.d.	0.24
	1,6,10-dodecatrien-3-ol	N.d.	N.d.	2.68	N.d.
	farnesol	N.d.	41.21	N.d.	19.33
	2-furanmethanol	N.d.	N.d.	N.d.	1.47
	L-α-bisabolol	1.52	N.d.	2.94	N.d.
Aldehyde		**31**	**22**	**N.d.**	**31**
	acetaldehyde	4.11	1.58	N.d.	N.d.
	2-hexenal	N.d.	1.27	N.d.	4.10
Ester		**0.4**	**14**	**14**	**10**
	acetic acid ethyl ester	N.d.	0.13	N.d.	N.d.
	butanoic acid, ethyl ester	1.46	N.d.	N.d.	N.d.
	hexanoic acid, methyl ester	N.d.	9.20	N.d.	1.57
	hexanoic acid, ethyl ester	N.d.	13.97	7.51	16.59
	acetic acid, hexyl ester	1.68	0.64	3.12	2.46
	octanoic acid, methyl ester	N.d.	0.40	N.d.	N.d.
	L-acetic acid, phenylmethyl ester	N.d.	N.d.	N.d.	1.01
	butanoic acid, hexyl ester	N.d.	N.d.	5.43	1.47
	octanoic acid, ethyl ester	N.d.	0.39	N.d.	1.32
	acetic acid, octyl ester	3.41	0.83	N.d.	N.d.
	propionic acid, ethyl ester	N.d.	N.d.	11.03	N.d.
	hexanoic acid, hexyl ester	N.d.	N.d.	N.d.	1.77
	butanoic acid, octyl ester	N.d.	0.33	N.d.	N.d.
	methoxyacetic acid, 2-tetrahydrofurylmethyl ester	N.d.	N.d.	N.d.	3.60
	hexanoic acid, dodecyl ester	N.d.	0.53	N.d.	N.d.
	1,2-benzenedicarboxylic acid, diethyl ester	0.87	N.d.	2.51	0.51
	phthalic acid, isobutyl-octyl ester	0.55	N.d.	N.d.	N.d.
	benzoic acid, 2-hydroxyphenylmethyl ester	N.d.	N.d.	0.85	N.d.
	2-hexen-1-ol, acetate	1.68	1.88	N.d.	1.99
	hexyl isovalerate	N.d.	N.d.	9.61	N.d.
	n-octyl 2-methylbutyrate	N.d.	0.52	N.d.	N.d.
	dihydromethyl jasmonate	3.77	N.d.	N.d.	0.58
	sulfurous acid, hexyl 2-pentyl ester	N.d.	N.d.	2.49	N.d.
Furan derivative		**0.4**	**0.2**	**N.d.**	**94**
	furan, 5-(1,5-dimethyl-1,4-hexadienyl)-2-ethyl-tetrahydro-2-methyl	0.5	0.27	N.d.	11.2
Ketone		**19**	**12**	**26**	**19**
	3-hexanone	N.d.	N.d.	N.d.	0.56
	2(3*H*)-furanone	9.01	16.44	7.51	24.94
	γ-decalactone	19.13	N.d.	28.01	N.d.
	γ-dodecalactone	N.d.	1.24	3.53	N.d.

Table 3 (Cont.)

Class	Compound	Cultivar			
		Fortuna	**Rubygem**	**Sweet Ann**	**Ventana**
Terpene		**34**	**0.4**	**15**	**0.9**
	linalool	N.d.	N.d.	4.28	N.d.
	L-α-terpinolene	3.3	6.18	N.d.	1.21
	geraniol	N.d.	0.94	N.d.	N.d.
	nerolidol	11.1	N.d.	24.48	15.17
	E-nerolidol	51.03	N.d.	0.78	N.d.
	trans-β-farnesene	N.d.	0.81	N.d.	N.d.

N.d. = not detected

Table 4. Volatile Compounds of Korean Black Raspberry Juice (Adapted from Lim *et al.*, 2012)

Volatile compounds	Juice (mg/L)
Acetaldehyde	26.64 ± 1.60
Acetone	1.97 ± 0.03
2-Butanol	125.18 ± 2.30
Damascenon	0.98 ± 0.0
Ethyl acetate	10.11 ± 0.42
Isoamyl alcohol	28.45 ± 1.05
Methyl acetate	4.61 ± 0.10
Methyl alcohol	114.56 ± 2.88
Total	**312.50 ± 8.34**

Table 5. Volatile Concentration (μg/kg) in Three Red Raspberry Fruit Cultivars Grown in Washington in 2005 (Adapted from Malowicki *et al.*, 2008)

Compound	Cultivars		
	Yellow Meeker	**Willamette**	**Tulameen**
δ-decalactone	470 ± 35	260 ± 24	510 ± 24
ethyl hexanoate	3 ± 2	3 ± 2	8 ± 0
geraniol	130 ± 7	48 ± 9	155 ± 3
2-heptanone	57 ± 24	90 ± 50	65 ± 4
hexanal	150 ± 35	110 ± 30	70 ± 5
(*E*)-2-hexenal	460 ± 200	250 ± 150	420 ± 36
(*Z*)-3-hexenol	180 ± 10	160 ± 27	86 ± 12
(*Z*)-3-hexenyl acetate	7 ± 1	15 ± 5	1 ± 0
α-ionone	63 ± 6	54 ± 4	20 ± 1
β-ionone	70 ± 3	73 ± 1	62 ± 2
4-isopropylbenzyl alcohol	36 ± 5	2 ± 2	2 ± 0
limonene	1 ± 1	1 ± 0	2 ± 0
linalool	16 ± 1	20 ± 3	140 ± 13
6-methyl-5-hepten-2-ol	55 ± 5	10 ± 0	29 ± 3
methyl nonanoate	N.d.	N.d.	1 ± 0
myrcene	10 ± 4	6 ± 4	6 ± 1
nerol	19 ± 1	4 ± 1	23 ± 2
2-nonanol	5 ± 1	2 ± 0	N.d.
2-nonanone	20 ± 2	13 ± 1	2 ± 0
δ-octalactone	390 ± 45	200 ± 40	600 ± 16
para-cymene	12 ± 2	1 ± 1	3 ± 0
α-phellandrene	50 ± 16	20 ± 4	20 ± 4
α-pinene	30 ± 4	22 ± 5	4 ± 0
sabinene	12 ± 10	4 ± 4	8 ± 1
terpinen-4-ol	120 ± 52	46 ± 6	74 ± 7
α-terpinene	15 ± 6	5 ± 2	10 ± 1
γ-terpinene	13 ± 8	4 ± 2	2 ± 0
α-terpineol	20 ± 2	17 ± 2	120 ± 12
terpinolene	3 ± 0	1 ± 1	3 ± 0

N.d. = not detected.

2-2. Berries and Health Effects

Many studies reported that consumption of berries reduces the risk of cardiovascular disease, metabolic syndrome and cancer because of their anti-oxidant and anti-inflammatory activities (Wang and Stoner, 2008; Prior and Gu, 2005; Folmer *et al*., 2014; Babu *et al*., 2013).

A. Anti-Cancer Effect of Berries

Folmer *et al.,* (2014) reported that phytochemicals derived from berries showed anti-inflammatory effects, increased carcinogen detoxicating enzymes, and modulated cell proliferation and apoptosis signaling pathways. The anti-cancer effect of anthocyanins from berries *in vitro* was studied by some cell culture models including colon (Lopez de las Hazas *et al*., 2016), liver (Do Thi and Hwang, 2018), breast (Amatori *et al.*, 2016) and leukemic cells (León-González *et al*., 2018). In these studies, anthocyanins showed anti-carcinogenic effects which include scavenging reactive oxygen species (ROS), increasing the oxygen-radical absorbance capacity, activating the expression of phase 2 detoxification enzymes, reducing lipid peroxidation, suppressing mutagenesis by carcinogens, and reducing cellular proliferation by regulating signal pathways (Wang and Stoner, 2008). Cranberry extract, rich in proanthocyanidins, increased apoptosis with cell cycle arrest in G1 and G2 phase (Nandakumar *et al.,* 2008). In addition, anthocyanins from cranberry showed suppression of cancer development in carcinogen-treated animals. *In vivo* studies showed that anthocyanins in food inhibited cancers of the gastrointestinal tract and applied anthocyanins suppressed skin cancer (Cooke *et al*., 2006; Wang and Stoner, 2008).

B. Anti-Metabolic Syndrome Effect of Berries

In the study of Basu *et al.* (2010), 48 participants with metabolic syndrome consumed freeze-dried blueberry beverage (50g freeze-dried blueberries, equivalent to ~350g or 2.3 cups

fresh blueberries) or fluids (control) every day for 8 weeks in a randomized controlled trial. Systolic and diastolic blood pressures were decreased more in the blueberry-supplemented group (-6 and -4%, respectively) than in control group (-1.5 and -1.2%) ($P = 0.003$ and $P = 0.04$). This study showed a protective role of blueberries in adults with metabolic syndrome, including a significant reduction in systolic and diastolic blood pressures, plasma oxidized LDL (ox-LDL) and lipid peroxidation. Other studies also reported that berry supplements using chokeberries, cranberries, or a combination of berries improved metabolic syndrome such as dyslipidemia, hypertension, and impaired fasting glucose (Erlund *et al*., 2008; Basu *et al.,* 2010). Morimoto *et al*. (2005) reported that raspberry ketone, a major fruity odor compound of red raspberry, prevented body weight increase induced from high-fat diet and activated lipid metabolism, improving obesity and fatty liver in mouse model with high-fat diet.

C. Anti-Diabetes Effect of Berries

According to Babu *et al*. (2013), consumption of berries and dietary anthocyanins showed effective reduction of type 2 diabetes mellitus risk. In an *in vitro* study about type II diabetes, they reported that Maqui Berry (MB) anthocyanins inhibited adipogenesis and suppressed inflammation (Schreckinger *et al.,* 2010). In another *in vivo* study, obese diabetic mice fed high fat diet with anthocyanins from Maqui Berry decreased fasting hyperglycemia. Especially D3S5G (Delphinidin-3,5-diglucoside), an abundant compound of MB anthocyanins, showed properties similar to insulin in muscle and liver cells and was responsible in part for the *in vivo* anti-diabetic effect of MB anthocyanins. (Rojo *et al.,* 2012). Blueberries have also been reported to show effects on metabolic syndrome and type 2 diabetes in animal-obesity models (DeFuria *et al*., 2009). In the study of DeFuria *et al*. (2009), supplementation of high-fat diet with whole blueberry powder (2.7% of the total energy in the diet) to male mice for 8 weeks

reduced inflammation in adipose tissue and insulin resistance. The extracts from the blueberry, rich in anthocyanins, showed hypoglycemic activity similar to metformin, an anti-diabetic drug (Lila, 2011). Paquette *et al.* (2017) investigated the effects of strawberry and cranberry polyphenols in insulin-resistant overweight or obese human subjects (n=41). Supplementation with 333 mg of strawberry and cranberry polyphenols for 6 weeks increased insulin sensitivity in overweight and obese insulin-resistant human subjects.

D. Anti-Cardiovascular Disease Effect of Berries

Consuming fruits and vegetables helps reduce the risk of cardiovascular diseases. McKay and Blumberg's study (2007) showed that cranberries contribute to reducing the risk of cardiovascular disease (CVD) through anti-inflammatory mechanisms. There is increasing evidence of anti-cardiovascular diseases effect from consumption of strawberries (Giampieri *et al.,* 2012). In a study of Jenkins *et al*. (2008), 28 of 85 hyperlipidemic patients with a cholesterol-lowering diet for 2.5 years were given a daily strawberry supplement (454 g/d) with a 2-week washout between two interventions as a 1-month randomized crossover substudy. As a result, strawberry supplementation increased the anti-oxidant effect by decreasing oxidized LDL, reduced the risks of coronary heart disease, and decreased blood lipids.

E. Anti-Inflammatory Effects of Berries

Recently, many studies have investigated cellular signaling, especially modification of inflammation pathways, of phenolic compounds (Joseph *et al*., 2016). In *in-vitro* study, whole berry extracts from blackberry, black raspberry, blueberry, cranberry, grape, raspberry, elderberry, pomegranate, and strawberry, showed anti-inflammatory effect by inhibiting LPS-induced NF-κB activation and the production of COX-2 and PGE_2 in RAW264.7 macrophage cell model (Folmer *et al.,* 2014). In Xie *et al*. (2011), blueberries reduced the production of pro-

inflammatory cytokines, such as TNF-α and IL-6, in LPS-stimulated RAW264.7 mouse macrophages via inhibition of NF-κB and mitogen-activated protein kinase (MAPK) pathways. Anthocyanins from berry extracts also exhibited significant inhibitory effects on production of NO (Wang and Mazza, 2002). Anthocyanins from blueberry, blackberry, and blackcurrant showed anti-inflammatory effects by inhibiting nuclear translocation of NF-κB independent of nuclear factor E2-related factor 2 (NRF2)-mediated mechanism in macrophages (Lee *et al*., 2014). Other studies also showed that berry compounds affected many signal transduction pathways through modulating inflammation-related regulatory genes such as AP-1, P1-3K/Akt, p38/Erk1/2, downregulating COX-2, and iNOS (Stoner *et al.,* 2007). Although there is no study related to the anti-inflammatory effect of whole berry volatile extracts, there are some anti-inflammation studies with purified volatile compounds, such as linalool and D-limonene, one of major natural terpene compounds in many flowers and plants with pleasant scent (Li *et al*., 2015; Lateef *et al*., 2014; Yu *et al*., 2017). Linalool (162, 324 and 648 μM), a natural compound of essential oils, showed anti-inflammatory effect on LPS-induced BV2 microglia cells through Nrf2/HO-1 signaling pathway (Li *et al.*, 2015). Huo *et al.* (2013) reported that 40, 80 and 120 μg/mL of linalool showed anti-inflammatory effect in LPS-induced RAW264.7 cells by suppressing pro-inflammatory cytokines such as TNF-α and IL-6 and blocking NF-κB and MAPK pathways. Yu *et al*. (2017) reported that D-limonene (50 and 100 mg/kg) consumption for 7 days showed anti-inflammatory effects in ulcerative colitis rat model (n=8/group) by modulating iNOS, COX-2, PGE2 and ERK signaling pathways. Consumption of D-limonene (50 mg/kg) for 28 days showed protective effect against diabetes by lowering blood glucose, LDL, total cholesterol and triglyceride levels in streptozotocin-induced diabetic rats (Bacanlı *et al*., 2017). Leu *et al.* (2017) reported that raspberry ketone, a major aromatic compound in raspberry

(300 μM, 160 mg/kg) inhibited lipid accumulation by modulating autophagy in 3T3-L1 cells and ovariectomy-induced obese rats. Jeong and Jeong (2010) found that 125, 250, 500 and 1000 μg/mL of rheosmin (raspberry ketone) isolated from pine needles showed anti-inflammatory effect in LPS-induced RAW264.7 cells by inhibiting NO, PGE_2, iNOS and COX-2 and blocking NF-κB pathways.

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CHAPTER 2: A Comparative Study of the Anti-Inflammatory Effects of Berry Phenolics and Volatiles

1. Abstract

Berries are rich in natural compounds, such as minerals, vitamins, dietary fibers, and especially phenolics and volatiles. Many studies found significant beneficial health effects of berry phenolics. However, there is limited information available on health-promoting activities of berry volatiles. The objective of this study was to investigate anti-inflammatory effect of phenolics and volatiles using a lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cell model by measuring the production of nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). RAW264.7 cells were pretreated with 3 different dilutions (50, 100, and 200-fold) of six berry (blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry) phenolic or volatile fractions for 1h. Cells were then incubated with or without LPS (100 ng/mL) for 24h. The NO production was measured by using Griess reaction. The level of IL-6 and TNF-α in the culture media were quantified using ELISA kit. Results showed that berry phenolics and volatiles significantly decreased the LPS-induced NO production by 38-93% and 47-79%, respectively ($P < 0.001$). There were no significant differences among three different dilutions of berry phenolics and volatiles in the RAW264.7 cells. Except black raspberry and red raspberry, berry volatiles decreased the level of TNF-α and IL-6 in LPS-stimulated RAW264.7 cells comparable to berry phenolics. These findings showed that volatiles from six common berries have comparable *in vitro* anti-inflammatory activity to berry phenolics by modulating the production of NO, IL-6, and TNF-α. Results from this study suggest that berry volatiles may be used as effective treatments to prevent inflammatory related diseases.

2. Introduction

Inflammation is the defense response from our body to fight infections, such as chemical stimuli and toxins (Xie *et al*., 2012; Qian *et al*., 2015). However, prolonged chronic inflammatory conditions, which shows characteristics of autoimmune diseases, can develop into human diseases, such as cancer (Huo *et al*., 2013; Dmitrieva *et al*., 2016), metabolic syndrome (Cani *et al*., 2007; Calder *et al*., 2011; Ouchi *et al*., 2011), diabetes (Shu *et al*., 2012; Agrawal and Kant, 2014), and cardiovascular diseases (Fearon and Fearon, 2008; Holvoet, 2008; Jang *et al*., 2016). This pathogenic inflammatory process is regulated by cytokines and pro-inflammatory factors, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Ahmed, 2011; Lee *et al*., 2016; Alvarez-Suarez *et al*., 2017). The pathogenic inflammation is increased by unregulated excessive production of pro-inflammatory factors and cytokines. Accordingly, these cytokines and pro-inflammatory factors can be used as important targets for the development of anti-inflammatory agents (Mantovani *et al*., 2008).

Many factors, including diet, lifestyle, and medication, can prevent or modify inflammatory processes and promote healthier life (Libby, 2006). Dietary components, especially natural compounds have been reported for many beneficial health effects, including effects on inflammatory, infectious, and chronic diseases (Jayaprakasha *et al*., 2013). Bioactive compounds from fruits and vegetables showed a number of health benefits in prevention of inflammation and chronic diseases (Patil *et al*., 2009; Jayaprakasha *et al*., 2013).

Berries, one of the most common fruits in the human diet, are rich in nutritive and non-nutritive natural compounds, such as minerals, vitamins, dietary fibers, and especially polyphenols (Battino *et al*., 2009; Afrin *et al*., 2016). The most common berries consumed in the

United States are blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberries (Basu *et al*., 2010). There are many studies that found significant beneficial health effects of berry polyphenols (Lim *et al*., 2013; Wang *et al*., 2013; Qian *et al*., 2015). Qian *et al*. (2015) reported that berry extracts, which contain many polyphenols, showed antioxidant (Skrovankova et al., 2015; Genskowsky *et al.*, 2016), anti-diabetic (Stote *et al.*, 2017), anti-obesity (Noratto *et al*., 2016) and anti-inflammatory activities (González-Gallego *et al.*, 2014; Chew *et al*., 2018). In *in-vitro* study, most berry extracts from blackberry, black raspberry, blueberry, cranberry, grape, raspberry, elderberry, pomegranate, and strawberry, inhibited NF-κB activation and the production of COX-2 and PGE_2 (Folmer *et al.,* 2014). However, Nile *et al*. (2014) pointed out that although the biological activities of the berry polyphenols were extensively studied in some reports, the activities of volatile compounds of berries were little known.

Berry volatiles are responsible for fruit flavor (Nile *et al*., 2014). Aroma is one of the most significant characteristics that affects the flavor of a fruit, its acceptability and consumption (Dymerski *et al.,* 2015; Fenoll *et al.,* 2009). Recently, it was suggested that flavor precursors of some volatile compounds may prevent or impede diseases, such as obesity (Park, 2015), type 2 diabetes (Flanagan *et al.*, 2014), and cancer (Linnewiel-Hermoni *et al.*, 2015). Although over 11,000 volatile components in foods were identified, there is still lack of research on volatile compounds in prevention of diseases (Ayseli and Ayseli, 2016). The objectives of this study are to investigate the anti-inflammatory effect of berry volatiles using a LPS-stimulated RAW264.7 macrophage cell model, by studying the modulation of production of NO, IL-6, and TNF-α.

3. Materials and Methods

3-1. Materials

Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals were of reagent grade. Frozen berries (blackberry, black raspberry, blueberry, red raspberry and strawberry) were obtained from local supermarkets. Frozen cranberries were purchased from Northwest Wild Foods (Burlington, WA).

3-2. Extraction of Berry Phenolics and Volatiles

Berry phenolics and volatiles were extracted by Dr. Howard's lab (University of Arkansas, Fayetteville, AR).

Berry Phenolic Extracts

The phenolic extracts were obtained by homogenizing 50 g of berries for 1 min in 50 mL of extraction solution containing methanol/water/formic acid (60:37:3 v/v/v) to the smallest particle size using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH). After centrifuging homogenates for 5 min at 10,000 rpm, the pellet was re-extracted with 50 mL of extraction solution containing acetone/water/acetic acid (70:29.5:0.5 v/v/v) and centrifuged again for 5 min at 10,000 rpm. Each of these extractions were repeated, and supernatants were dried in a rotary evaporator at 40°C. The combined dried extract was reconstituted in 50 mL deionized water to be further purified with solid phase extraction. Solid phase extraction was performed with Sep-Pak C_{18} 20 cc Vac Cartridge, 5 g Sorbent per Cartridge, 55-105 μm particle size (Waters Corp, Milford, MA). The cartridges were activated by eluting 40 mL methanol, followed

by 80 mL water. The crude phenolic extract was loaded onto the column and rinsed with 60 mL of water. The purified phenolic extracts were eluded with 80 mL of 70% ethanol. The eluent was dried with a rotary evaporator at 40˚C and reconstituted with 50 mL deionized water.

Berry Volatile Extracts

Volatiles were obtained by combining 200 g of frozen berries, 200 mL deionized water, and 100 g NaCl and blending for 1 min in a Waring blender. The slurry was vacuum distilled at 28 in. Hg (50°C) for 30 min and 250 mL of the volatile extracts were collected in a flask contained in an ice water bath.

3-3. Cell Culture and Treatment

The murine macrophage cell line RAW 264.7 (ATCC® TIP-71) was purchased from American Type of Culture Collection (ATCC, Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% sodium bicarbonate and 1% penicillin (100 U/ml)-streptomycin (100 µg/mL). The cells were then incubated in an atmosphere of 5% CO_2 at 37°C in humidification incubator (VWR® Water Jacketed CO2 incubator, VWR International, PA) and were subcultured every 2 days. For the experiments, RAW 264.7 cells were pretreated with three different concentrations (diluted 50, 100, and 200-fold) of berry phenolics or volatiles (blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry). We diluted berry phenolics or volatiles dissolved in Tween 80 (0.03% final concentration) with DMEM before treatment.

3-4. Nitric Oxide (NO) Measurement

The effect of berry phenolics and volatiles on LPS-stimulated NO production was measured by using Griess reaction (Green *et al*., 1982). The Griess reagent system (Promega

Co., Madison, WI) is based on the chemical reaction with sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. RAW 264.7 cells were plated at a density of 1.0 X 10^4 cells/well in 96-well plates containing 100 µL of medium, and then incubated in a 37°C, 5% CO_2 incubator overnight (16 h). After overnight incubation, the cells were pretreated with three different dilutions of six berry phenolics or volatiles (diluted 50, 100, and 200-fold) for 1 h and the treatment was removed and washed twice with working media. The cells were then stimulated with LPS (100 ng/mL) for 24 h. After 24 h, culture supernatants were collected to measure nitrite content. Equal volumes (50 µL) of Griess reagent (Promega Co., Madison, WI) and sample were incubated together at room temperature for 10min (1:1 of 0.1% N-1-napthylethylendiamine dihydrochloride in water and 1% sulfanilamide in 5% phosphoric acid). Absorbance at 540 nm was measured using a microplate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc. Winooski, VT). Fresh culture media was used as a blank in all experiments. Nitrite production, an indicator of NO synthesis, were calculated against a sodium nitrite standard curve.

3-5. IL-6 and TNF-α Measurement

After pretreatment of 50-fold dilution of six berry phenolic or volatile fractions for 1 h and stimulation with LPS (100 ng/mL) for 24 h, levels of IL-6 in the cell culture supernatants were quantified by using RayBio® Mouse IL-6 Enzyme-linked immunosorbent assay (ELISA) Kit (RayBiotech Inc., Norcross, GA), according to the manufacturer's instructions. Concentration of TNF- α in the cell culture supernatants were measured using RayBio® Mouse TNF-alpha ELISA Kit (RayBiotech Inc., Norcross, GA) according to manufacturer's protocol.

3-6. Statistical Analysis

All statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Data were presented as mean ± Standard Error of the Mean (SEM). All experiments were conducted in at least triplicate, and significant differences among the groups were determined using a one-way analysis of variance (ANOVA) and Tukey's test at 5% level of significance ($P < 0.05$).

4. Results

4-1. Effect of Berry Phenolics and Volatiles on LPS-Induced NO Production

To determine whether six berry phenolic or volatile treatments affect the production of NO, we investigated their inhibitory effects on LPS-induced NO production in RAW 264.7 murine macrophage cells. In both (A) and (B) in Figure 2, treatment of RAW 264.7 cells with LPS alone significantly increased in NO production compared with the control group ($P < 0.001$). However, both berry (A) phenolics and (B) volatiles (50, 100, and 200-fold dilution) significantly decreased the LPS-induced NO production by 38-93% and 47-79%, respectively ($P < 0.001$). There was no significant difference between the effect of phenolics and volatiles on LPS-induced NO production within each dilution in each berry, except in blueberry 50 and 200-fold dilution. The result also showed that there were no significant differences among three different dilutions (50, 100, and 200-fold dilution) within each berry phenolic and volatile treatment, except between 100-fold dilution and 50 and 200-fold dilution in cranberry and blueberry. Therefore, we decided to use only 50-fold dilution berry phenolic and volatile treatments for TNF-α and IL-6 ELISA assay.

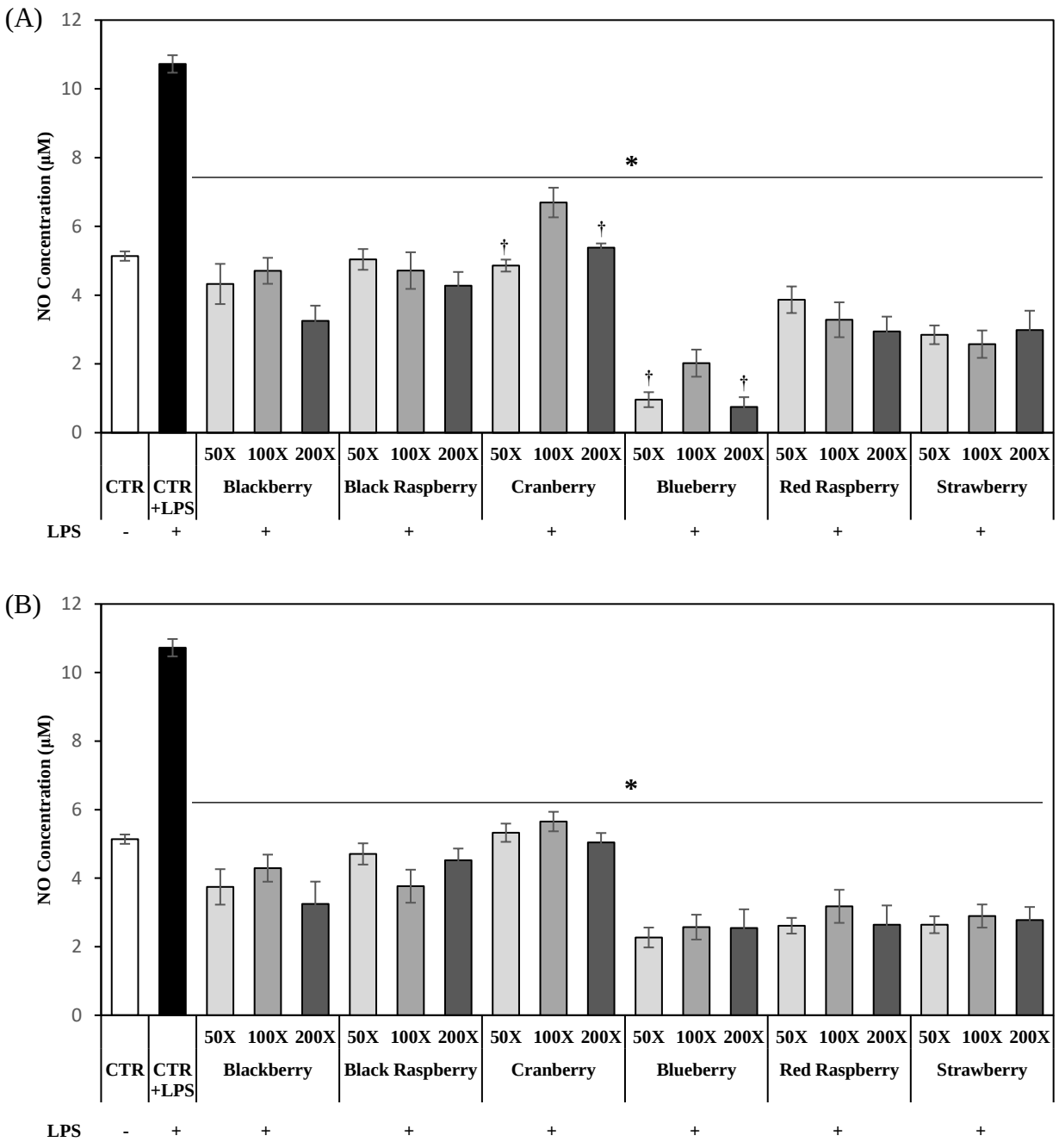

Figure 2. Effect of (A) Berry Phenolics and (B) Volatiles on LPS-Induced NO Production. Data are expressed as Mean ± SEM (n = 10). * indicates significant difference compared to LPS-stimulated control at $P < 0.001$. † indicates significant difference compared to 100-fold dilution within each berry phenolic treatment ($P < 0.05$).

4-2. Effect of Berry Phenolics and Volatiles on LPS-Induced Pro-Inflammatory Cytokines Production

To further analyze the anti-inflammatory effect of six berry phenolics and volatiles, the production of pro-inflammatory cytokines (TNF-α and IL-6) was determined. In Figure 3, treatment of RAW 264.7 cells with LPS significantly induce LPS-stimulated TNF-α production ($P < 0.01$). Except black raspberry phenolics and volatiles and red raspberry phenolics, all treatments significantly decreased the level of TNF-α in LPS-stimulated RAW 264.7 cells ($P < 0.01$). In addition, all six berry volatiles showed comparable or higher inhibiting effect on LPS-induced TNF-α production to berry phenolics ($P < 0.05$). In Figure 4, treatment of RAW 264.7 cells with LPS significantly increased the level of IL-6 in LPS-stimulated RAW 264.7 cells ($P < 0.01$). Except black raspberry volatiles and red raspberry phenolics and volatiles, all phenolic and volatile treatments showed significant decrease in the level of IL-6 in LPS-stimulated RAW 264.7 cells ($P < 0.01$). In addition, all six berry volatiles except black raspberry volatiles showed comparable or stronger inhibiting effect on LPS-induced IL-6 production to berry phenolics ($P < 0.05$).

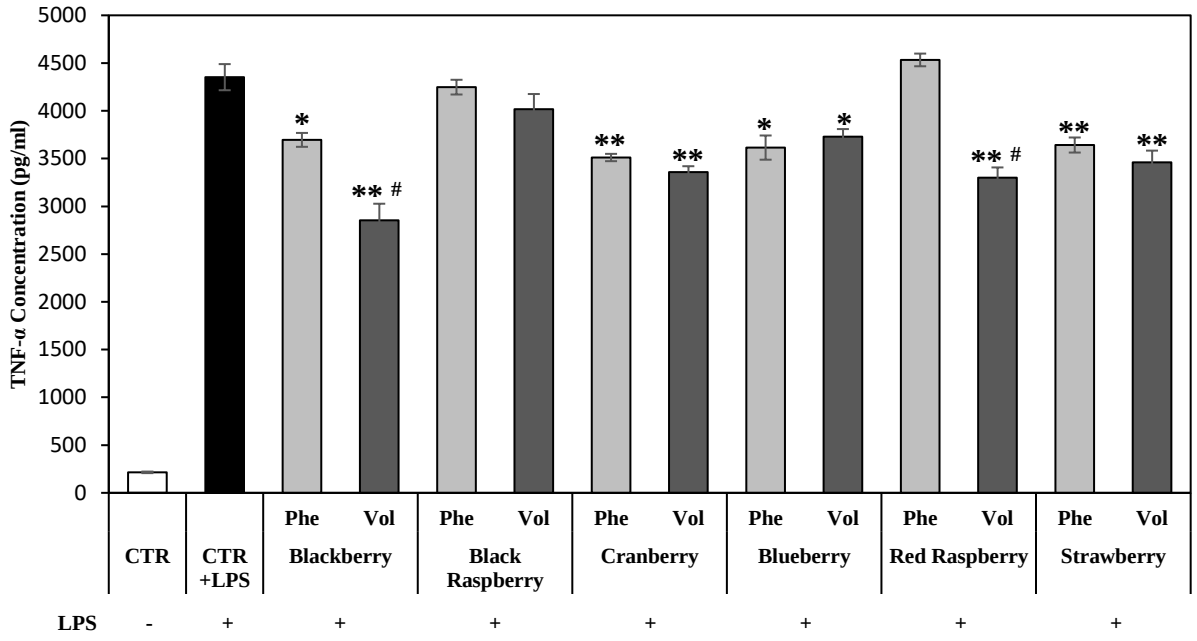

Figure 3. Effect of Six Berry Phenolics and Volatiles (50-Fold Dilution) on LPS-Induced TNF-α Production. Data are expressed as Mean ± SEM (n = 5). Significant difference was compared with LPS-stimulated control at $*P < 0.01$, $**P < 0.001$. $^{\#}$ indicates significant difference compared between berry phenolic and volatile treatment within each berry ($P < 0.05$).

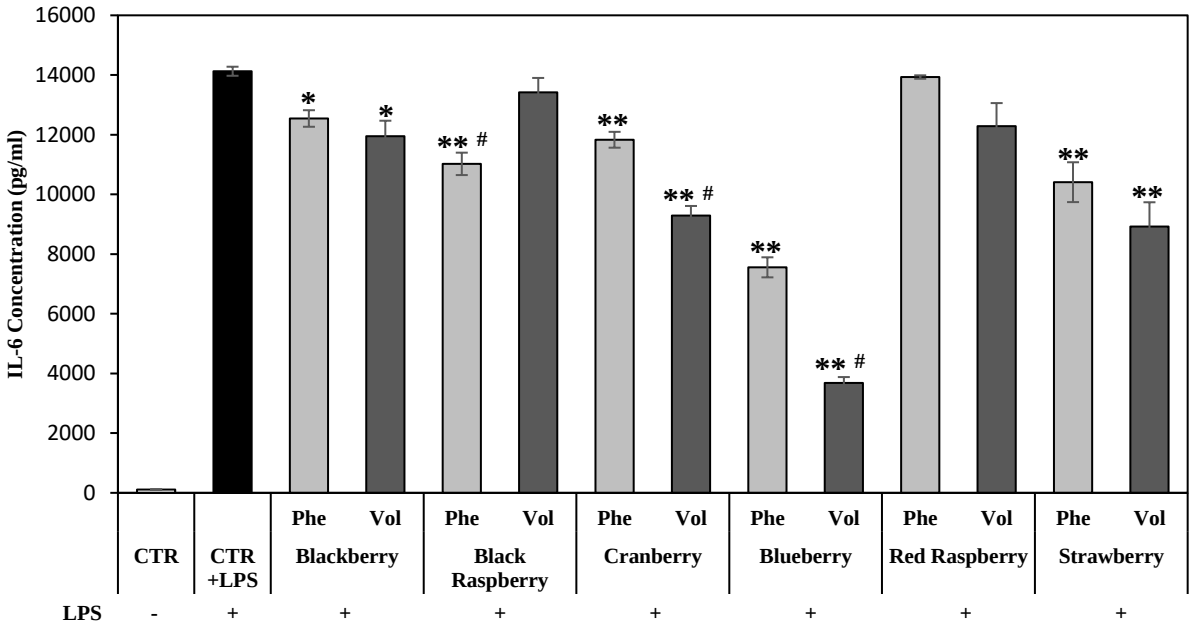

Figure 4. Effect of Six Berry Phenolics and Volatiles (50-Fold Dilution) on LPS-Induced IL-6 Production. Data are expressed as Mean ± SEM (n=5). Significant difference was compared with LPS-stimulated control at $*P < 0.01$, $**P < 0.001$. $^{\#}$ indicates significant difference compared between berry phenolic and volatile treatment within each berry ($P < 0.05$).

5. Discussion

Many health benefits from consumption of fruits and vegetables have been increasingly studied in the last two decades (Giampieri *et al*., 2015). Among fruits and vegetables, berries, a common fruit in human diet, showed notable health beneficial effects because berries have abundant levels of both nutritive (vitamins and minerals) and non-nutritive compounds, including phenolic and volatile compounds (Battino *et al*., 2016). In many different health beneficial effects of berries, researches about anti-inflammatory effect of berry extracts or berry phenolic compounds have increased (Joseph *et al*., 2016). However, it is hard to find studies about anti-inflammatory effects of berry volatiles, to our best knowledge. Berry volatiles represent the aroma and flavor of berries and they are widely used for processed food products, cosmetics and others (Dymerski *et al.,* 2015). Thus, the purpose of this study was to investigate anti-inflammatory effect of berry volatile extracts through the regulation of pro-inflammatory cytokines, comparing to berry phenolic extracts.

In inflammation, macrophages in the immune system crucially work as the host defense (Lee *et al*., 2016). LPS stimulates macrophages and make them produce many pro-inflammatory mediators such as NO, and other pro-inflammatory cytokines (IL-6 and TNF-α) (Heo *et al*., 2018). A lipophilic free radical, NO, maintains homeostasis in biological systems in our body (Bogdan, 2015). However, if there are inflammatory stimuli, high levels of NO are produced by inducible nitric oxide synthases (iNOS), unlike other two different isoforms of NOS that secrete low levels of NO (Földes *et al*., 2016). Overproduced NO can damage tissues and create reactive nitric oxygen species, thus accelerating inflammation indirectly (Guzik *et al*., 2002; Soufli *et al*., 2016). Anthocyanin from berry extracts exhibited significant inhibitory effects on production of NO (Wang and Mazza, 2002). Li *et al*. (2014) also found that crude extracts from black

raspberry and red raspberry (100, 150 and 200 μg/mL) significantly suppressed the level of NO production on LPS/IFN-γ-activated RAW264.7 macrophage cells. We found that both berry phenolics and volatiles significantly inhibit the excessive production of NO in LPS-stimulated RAW264.7 murine macrophage cells (Figure 2). Especially NO production in LPS-induced RAW264.7 cells significantly decreased in those with blackberry, blueberry, red raspberry and strawberry phenolics and volatile extract treatments, compared to control without LPS treatment ($P<0.05$). In many studies, phenolic extracts from berries showed no cytotoxic effect in RAW 264.7 macrophage cells (Li *et al*., 2015; Qian *et al*., 2015) and we also previously tested cytotoxicity of all berry phenolic and volatile extracts in RAW264.7 cells. As the result, there was no cytotoxic effect of berry phenolic and volatiles. In addition, there were no significant differences among three different dilutions (50, 100, and 200-fold dilution) so we chose 50-fold dilution berry phenolic and volatile extracts for TNF-α and IL-6 ELISA assays.

After LPS stimulation, macrophages also produce pro-inflammatory cytokines such as IL-6 and TNF-α (Cheng *et al*., 2015). IL-6 is a key cytokine which can directly potentiate many diseases such as myeloma and plasmacytoma (Kishimoto and Tanaka, 2015). In the initial inflammatory stage, IL-6 is synthesized and increases other cytokines and acute phase proteins rapidly by stimulating inflammatory signaling pathways including NF-κB (Fielding *et al*., 2014; Tanaka *et al*., 2014). TNF-α also plays an important role in inflammation. TNF-α induces other pro-inflammatory cytokines including IL-6 and activates inflammatory gene expression by using its receptors (Yeganeh Montakhab *et al*., 2017). Then, TNF-α increases the pathogenesis of inflammatory bowel disease, rheumatoid arthritis and asthma (Soromou *et al*., 2012; Kim *et al*., 2016). Thus, targeting IL-6 and TNF-α can be a beneficial prevention method to inhibit chronic inflammation (Wu *et al*., 2015). Cheng *et al*. (2014) showed that polyphenols from blueberries

inhibited inflammation by modulating pro-inflammatory cytokines such as IL-1β, IL-6, and IL-12. Parelman *et al*. (2012) also indicated that dietary strawberry powder is important for modulating inflammation by regulating pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in lean C57BL/6 mice. Black Satin and Jumbo cultivar extracts from blackberry (50 and 100mg/mL) significantly downregulated IL-6 gene expression and slightly decreased NO production on LPS-stimulated RAW264.7 cells (Van de Velde *et al*., 2016). In this study, all berry phenolics and volatiles except black raspberry and red raspberry showed significant suppression in TNF-α and IL-6 in LPS-stimulated RAW264.7 macrophage cells. As similar to our findings, Kim *et al*. (2013) reported that polyphenol of black raspberry (1, 5, 10, 25, 50 and 100 μg/mL) showed significant inhibition in NO and IL-6 production but there was no significant suppression in TNF-α production. Li *et al*. (2014) reported that anthocyanin-rich fractions from red raspberry (150 and 200 μg/mL) significantly suppressed IL-6 mRNA expression, which somewhat contradicts our findings. Cyanidins in black raspberry also inhibited inflammation in LPS-induced murine macrophage cell model by reducing pro-inflammatory cytokines including TNF-α and IL-6 (Jo *et al*., 2015). The study of Li *et al*. (2014) and Jo *et al*. (2015) showed significant anti-inflammatory effect with more fractionated and purified phenolic compounds. It indicates that higher concentration of more purified phenolic compounds or phenolic fractions from berries will be more effective in inhibiting pro-inflammatory mediators and cytokines. Even though some phenolics and volatiles from black raspberry and red raspberry were not significantly suppressed TNF-α and IL-6, they showed slight inhibiting effect with low concentrations. Thus, our findings in the present study found that all berry phenolics and volatiles have potential anti-inflammatory effect by reducing the level of NO, IL-6 and TNF-α in LPS-induced RAW264.7 macrophages, and it suggests that there will be more suppression effect

with purification or high concentration of berry phenolics and volatiles. These results confirmed previous findings for berry phenolics, but this is the first report that berry volatiles possess anti-inflammatory effect.

6. Conclusion

Berry phenolics and volatiles significantly decreased the LPS-induced NO production by 38-93% and 47-79%, respectively ($P < 0.001$) and there were no significant differences between the anti-inflammatory effect of berry phenolics and volatiles in the RAW264.7 cells. Except black raspberry and red raspberry, berry volatiles decreased the level of TNF-α and IL-6 in LPS-stimulated RAW264.7 cells, comparable to berry phenolics. These findings showed that volatiles from six common berries have comparable anti-inflammatory activity to berry phenolics by modulating the production of NO, IL-6, and TNF-α *in vitro*. Results from this study suggest that berry volatiles may be used as effective treatments to prevent inflammatory related diseases.

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Conclusion

The present study showed that berry phenolics and volatiles significantly inhibited the level of NO production in LPS-induced RAW264.7 cells. Except black raspberry and red raspberry, berry phenolics and volatiles reduced the level of TNF-α and IL-6 in LPS-stimulated RAW264.7 cells. There were no significant differences between the anti-inflammatory effect of berry phenolics and volatiles in the LPS-activated RAW264.7 cells. These results demonstrated that volatiles from six common berries have anti-inflammatory effect which is comparable to berry phenolics by modulating the production of NO, IL-6, and TNF-α *in vitro*. This research suggest that berry volatiles may be beneficial for preventing inflammation and inflammatory related diseases.

Appendix

IBC Protocol Approval Letter

December 7, 2016

MEMORANDUM

TO: Dr. Sun-Ok Lee

FROM: Ines Pinto, Biosafety Committee Chair

RE: Protocol Modification

PROTOCOL #: 16011

PROTOCOL TITLE: Testing bioactive components (arachidin-1 and 3, conjugated linoleic acid, saponins, berry volatiles) for proliferation and inflammatory responses

APPROVED PROJECT PERIOD: Start Date September 10, 2015 Expiration Date September 9, 2018

The Institutional Biosafety Committee (IBC) has approved your request, dated December 5, 2016, to modify Protocol # 16011, "Testing bioactive components (arachidin-1 and 3, conjugated linoleic acid, saponins, berry volatiles) for proliferation and inflammatory responses".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

Form 1: GENERAL INFORMATION, contd

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name: (first and last) - POSITION (Title, academic degrees, certifications, and material field ofexpertise)	**QUALIFICATIONS/TRAINING/RELEVANT EXPERIENCE Describe previous work or training with biohazardous and/or recombinant DNA and include Biosafety Levels)**
Example: Bob Biohazard - Associate Professor, PhD Microbiology	14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.
Sun-Ok Lee - Assistant Professor	16 years of experience in conducting research involving human cell and animal tissue cultures
Cindi Brownmiller - Research Associate	12 months of experience in cell cultures
Lacy Nelson-Program Technician II	No experience. Get a training from Dr. Lee
Inah Gu- M.S. student	5 months of experience in cell cultures
Macy Shirley - Undergraduate student	No experience. Get a training from Dr. Lee
Danielle Ashley- M.S. student	No experience. Get a training from Dr. Lee
Wing Shun Lam- M.S. student	No experience. Get a training from Dr. Lee
Brittany Frederick- M.S. student	No experience. Get a training from Dr. Lee

Additional Personnel Information (if needed):

All personnel took the EH&S Online training including bloodborn pathogens, hazardous waste, biosafety, fire safety, and autoclave safety.
All personnel are provided with the hepatitis A & B immunizations. The use of departmental cost center is allowable. It will keep a log as whom have received it and whom have declined.