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Anti-inflammatory Activity of Phenolic and Volatile Compounds in Cranberries (Vaccinium macrocarpon L)

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Anti-inflammatory Activity of Phenolic and Volatile Compounds in Cranberries (*Vaccinium macrocarpon* L*)*

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by

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Abstract

The primary objective of this study was to compare the anti-inflammatory effects of phenolic and volatile compounds extracted from cranberries. The Griess Reagent System assay was used to measure the *in vitro* anti-inflammatory capabilities of cranberry phenolic and volatile extracts on RAW 264.7 mouse macrophage cells. This study tested the anti-inflammatory capabilities of the cranberry phenolic and volatile extracts before, as a preventative treatment, and after, as a means of treating pre-existing inflammation, inducing inflammation with lipopolysaccharide (LPS). All experiments were conducted in the following manner, varying only in whether treated with the extracts before or after LPS: 1 x 10^3 RAW 264.7 cells were seeded into individual wells of a 96 well plate, given 16 hr to attach, and treated with the phenolic and volatile extracts at 2x, 4x, and 8x dilutions of their respective starting concentrations present in a cranberry for 1 hr either before or after 24 hr of induced inflammation by LPS. Then, nitric oxide (NO) levels were measured to assess the anti-inflammatory capabilities. When treated with the extracts after LPS, the phenolic 635.7 ppm, 317.8 ppm and volatile 1.8 ppm NO levels were significantly lower than the positive control, reduced by 62%, 46%, and 50% respectively. When treated before LPS, the phenolic 635.7 ppm, 317.8 ppm and volatile 1.8 ppm, 0.9 ppm NO levels were significantly lower than the positive control, reduced by 52%, 25%, 47%, and 13% respectively. Upon overall evaluation, the phenolic and volatile extracts' anti-inflammatory capabilities were very comparable even though the volatiles were at a 353x lower concentration, and an overall stronger preventative effect was observed. Future studies are needed to reveal the mechanisms by which these compounds act to prevent and reduce inflammation and to determine the bioavailability of these compounds.

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Chapter 1: Introduction and Objectives

1.1 Introduction

Inflammation is the human body's urgent response to tissue damage caused by chemicals, physical injury, or pathogens (Weiss 2008). Inflammation occurs very often in humans because humans are partly microbial and living in a microbial world, which results in countless interactions with microbial stimuli and the possibility of tissue damage occurring (Nathan and Ding 2010). The body's first line of defense to this tissue damage is called the acute inflammatory response, which is a non-specific response that mobilizes cells of the immune system, including macrophages, monocytes and neutrophils (Kumar et al. 2004). In a normal, healthy response, these immune cells arrive to the area of damage, activate the inflammatory response, and initiate an immune response that usually results in removal of the stimulus and healing of the tissue back to its' normal functioning state (Liddiard 2011). When the acute inflammatory response does not resolve, that is where problems arise. Non-resolving inflammation, called chronic inflammation, is an extended, unregulated and maladaptive response involving active inflammation, attempts at tissue repair, and tissue damage (Weiss 2008). Long-term low-grade chronic inflammation, noted by abnormally high levels of certain markers in the circulation including C-reactive protein, tumor necrosis factor-α, and interleukin-6, is a risk factor for several chronic diseases (Nicklas et al. 2005). Chronic inflammation does not directly cause obesity, cancer, asthma, obstructive pulmonary disease, multiple sclerosis, or rheumatoid arthritis, but it is known to significantly contribute to their and many other diseases' pathogenesis (Nathan and Ding 2010). Destructive periodontal disease, which affects the tissues surrounding and supporting the teeth, is another specific example of a disease involving inflammation. These patients, presenting with swollen red gums, have elevated levels of the

inflammatory mediators tumor necrosis factor-α and interleukin-6 (Khosravi et al. 2013). Obesity is another example of a disease involving inflammation and is arguably the most relevant to people in the United States. According to the most recent report by the Center for Disease Control and Prevention an overwhelming 36.5% of United States adults are obese (Ogden et al. 2015). The inflammation associated with obesity is caused by decreased insulin sensitivity, increased intracellular stress, increased autophagy, hypoxia, and apoptosis, resulting in the release of pro-inflammatory signals (Kloting and Bluher 2014).

Currently, several of the treatments for chronic inflammation include taking drugs such as ibuprofen (Manish et al. 2003), drugs that inhibit the toll-like receptor pathway (Lucas and Maes 2013) and use of immunocytokines (Bootz and Neri 2016). There are other types of behavioral treatments, involving diet and exercise training, where the patients do not take any drugs, but these are newer treatments and still an active area of investigation. Nicklas et al. (2005) hypothesized that increased levels of adipose tissue contribute to chronic inflammation. They summarized and reviewed 18 published studies on the topic and found that every study concluded that dietary restriction leading to weight loss reduced markers of chronic inflammation, including tumor necrosis factor-α, C-reactive protein, interleukin-6, interleukin-8, interleukin-18, and others. Lee et al. (2013) corroborated that adipose tissue is an important regulator of inflammation, releasing pro-inflammatory, TNF-α IL-1 IL-6 and IFN-γ, and anti-inflammatory cytokines, IL-4 IL-10 IL-3 and IL-Ra. They also stated that obesity, where a person contains excessive amounts of adipose tissue, is an underlying condition for inflammatory diseases (2013).

Diet is a critical factor in determining the level of adipose tissue, suggesting that diet plays a role in inflammation. Other studies that look at the specific effects of diet on

inflammation concluded that diets high in omega-3 fatty acids, fruits, nuts, vegetables, and whole grains are associated with lower levels of inflammation generated by production of anti-inflammatory cytokines, whereas diets high in refined starches, sugar, and fats are associated with higher levels of inflammation generated by excessive production of pro-inflammatory cytokines (Giugliano et al. 2006). Glisan et al. (2016) also noted that high fat diets were coupled with increased toll-like receptor 4 expression and nuclear factor κB activation in monocytes, which triggers the inflammatory response. New methods of prevention and treatment of inflammation are an active area of investigation in the field, and the data from previous studies strongly suggest that diet plays a role in inflammation (Nicklas et al. 2005) (Lee et al. 2013), and that certain dietary factors can act as preventative or treatment methods to lower inflammation.

Fruits and vegetables have a variety of compounds associated with health benefits. Cranberries (*Vaccinium macrocarpon* L) specifically, contain many bioactive compounds known to improve urinary tract health, boost immune function, and reduce cardiovascular disease (Kresty et al. 2011). Recent research also shows cranberries have potential anti-inflammatory effects. In a study of hepatic inflammation in obese mice, cranberry polyphenol-rich extract decreased inflammatory markers tumor necrosis factor-α and chemokine ligand 2 by 28% and 19% respectively (Glisan et al. 2016). The polyphenol-rich extract also decreased mRNA expression in the liver of the toll-like receptor 4 and nuclear factor κB, which are important in signaling pathways triggering inflammation, by 63% and 24% respectively, all over a period of ten weeks (2016). A study by Anhe et al. (2015) also showed anti-inflammatory effects of cranberry extract, specifically looking at the phenolic compounds, in high fat/high sucrose fed mice. Results showing that the cranberry extract fully prevented diet-induced intestinal inflammation in the mice by reducing COX2 activity and tumor necrosis factor-α. Both of the

aforementioned studies evaluated the cranberry extract for phenolic compounds, which likely have a role in the anti-inflammatory action of the extracts, but it is quite possible that other compounds in cranberries play a role in the berries' anti-inflammatory effect. This possibility presents the purpose of this study, to explore whether the volatile compounds in cranberries also have anti-inflammatory properties. There has been very little research conducted specifically on the anti-inflammatory properties of volatile compounds extracted from cranberries, but previously conducted research suggests that volatile compounds overall have anti-inflammatory and therapeutic potential due to their ability to suppress nuclear factor κB (Salminen et al. 2008). Previous research also indicates that individual volatile compounds in cranberries can act as possible anti-inflammatory agents. α-terpineol, which is the most abundant individual volatile compound found in cranberries, has already shown the ability to inhibit the nuclear factor κB pathway (Hassan et al. 2010). This previous evidence regarding volatile compounds and the most abundant individual volatile compound in cranberries, α-terpineol, gives the hypothesis for the current study encouraging potential, that cranberry volatile and phenolic compounds reduce inflammation on RAW 264.7 cells, *in vitro*.

1.2 Objectives

Objective 1: Extract, quantify and identify the volatile and phenolic compounds in cranberries via GC and HPLC methods.

Objective 2: Compare the anti-inflammatory effect of the volatile and phenolic extracts isolated from cranberries on RAW 264.7 mouse macrophage cells.

Objective 3: Compare the anti-inflammatory effect of individual, more abundant volatile compounds from cranberries (α-terpineol, eucalyptol, linalool oxide, and linalool) on RAW 264.7 mouse macrophage cells.

Chapter 2: Literature Review

2.1 Phenolic Compounds

2.1.1 Functional Properties of Phenolic Compounds

Phenolic compounds are found in abundance in fruits, plants, herbs, vegetables, seeds, roots, and leaves (Soto et al. 2015). In plants, the phenolics' main role is to protect against stress, but they also have a role in development, structural integrity and scaffolding, and plants' ability to adapt to changing environments (Bhattacharya et al. 2010). Phenolic compounds are secondary plant metabolites, produced in response to stressors such as ultraviolet radiation, ozone, pollution, infection by pathogens, extreme temperature exposure, and tissue damage (Soto et al. 2015). Phenolics also contribute to the color, taste, and putative health promoting benefits associated with the plant producing them (Boudet 2007). Phenolic esters play a role in strengthening the cell wall of plants, giving texture to plant foods, and contributing to plant disease resistant properties (Beveridge et al. 2000) (Parr et al. 1997). The total amount of phenolic compounds found in a particular plant depends on factors such as growing conditions, cultivation techniques, cultivar, ripening processes, storage conditions, and others, and the phenolic content may change, increasing under the stressful conditions previously mentioned (Soto et al. 2015).

2.1.2 Classification of Phenolic Compounds by Chemical Structure

Phenolic compounds contain one or more aromatic rings and one or more hydroxyl substituents by definition. These compounds are usually not found as free compounds (aglycones), they are most commonly esters, glycosides, or methyl esters (Soto et al. 2015). A "simple phenol" is a phenolic compound containing one aromatic ring, whereas a "polyphenol"

contains multiple aromatic rings, and the two groups can be further classified by their side chain groups.

Phenolic compounds can be formed through two different pathways, the acetic acid pathway and the shikimic acid pathway, forming mainly simple phenols and phenylpropanoids respectively (Soto et al. 2015). The "simple phenols" group can be further broken down into two groups, coumarins and phenolic acids. Coumarins are formed by the cyclization of *o*-coumaric acid and are glycosides. The phenolic acids contain one aromatic ring and a carboxylic acid side chain and they, along with their derivatives, are known for being native antioxidants (Koroleva et al. 2014). Caffeic acid, a specific phenolic acid derivative, is one of the most prevalent phenolic acids found in fruits and vegetables (Ghulam et al. 2014).

2.1.3 Bioavailability of Phenolic Compounds

Phenolic compounds have been extensively studied *in vitro*, but it is still questioned how and whether the effects seen in *in vitro* translate *in vivo*. The most common phenolic compounds in nature are phenolic acids and flavonoids therefore the bioavailability of phenolic compounds can be split into groups, phenolic acids and flavonoids (Karakaya 2010). Ferulic, sinapic, *p*-coumaric, and caffeic acids are all hydroxycinnamic acids, which is a subclass of phenolic acids, and have been found to have 25% absorption in the foregut after two hr, with a suggested mechanism of Na^+ dependent carrier-mediated transport for absorption (Wolffram et al. 1995). In another study with rats fed specifically a ferulic acid rich diet, 45-53% of the dose was reported available for peripheral tissues, and a proportion between urinary excretion of ferulic acid and ferulic acid dose was determined, leading to the conclusion that the compound was highly absorbed (Adam et al. 2002). However, it was determined that the when the ferulic acid was in a food with a complex matrix, such as a cereal, there was lower absorption of the compound

(Adam et al. 2002). The bioavailability of flavonoids used to be believed as negligible due to being bound to glycosides, and that only aglycones could pass into the bloodstream from the gut, however, now several studies have demonstrated that specific flavonoids have a much higher bioavailability than previously thought. Quercetin has been reported at 62% dose absorption in the bile twenty minutes after ingestion in Wistar rats (Crespy et al. 2002). In a human study by Hollman et al. (1995), orally administered quercetin absorption was approximately 24% and the absorption of quercetin glycosides from onions, which were previously thought to be negligible, was 52%.

2.2 Volatile Compounds

2.2.1 Functional Properties of Volatile Compounds

Volatiles are called "volatile" because they can be easily vaporized at ambient temperature. At ambient temperature they have a very low boiling point, or high vapor pressure. Fruits and vegetables produce a wide range of volatile compounds that make up their aroma, add to their flavor, and indicate the quality of the flavor (El Hadi et al. 2013). Although many of the same specific individual volatile compounds are found in different fruits and vegetables, it is the mixture of the volatile compounds that gives each fruit or vegetable its distinctive aroma (Tucker 1993). Volatile compounds also serve as a defense mechanism against insect feeding in plants. In response to mechanical damage, the compounds are synthesized and released to ward off insects (Pare and Tumlinson 1999). There are several different factors that affect the volatile composition of a fruit or vegetable, including degree of maturity, genetic makeup, postharvest handling and storage, and environmental conditions (El Hadi et al. 2013).

Volatile compounds are synthesized as secondary metabolites in fruits and vegetables, they are formed due to enzymatic activity when the tissue is disrupted due to cutting and

shredding (Gary 1999). For example, when chopping an onion, the smell is not very noticeable until the onion is sliced open, i.e. the tissue is disturbed, and the volatile compounds are formed and released. The volatiles important for the aroma and flavor of fruits and vegetables are biosynthesized from the following precursors: membrane lipids, amino acids, and carbohydrates (Sanz et al. 1997).

2.2.2 Classification of Volatile Compounds by Chemical Structure

Biosynthetic pathways for the synthesis of volatile compounds can begin with fatty acids, amino acids, carotenoids, and terpenoids. Terpenoids are the largest class of secondary plant metabolites and are just one of the groups contributing to the total volatile profile. They consist of one or more isoprene units forming a hydrocarbon chain, and several variations including oxygenated, acyclic, monocyclic, and bicyclic exist. Terpenoids are commonly broken down into classes by the number of isoprene units in their structure. Hemiterpenoids (1 isoprene unit and 5 carbon atoms), monoterpoids (2 isoprene units and 10 carbon atoms), sesquiterpoids (3 isoprene units and 15 carbon atoms), homoterpoids (2 and 3 isoprene units and 11 and 16 carbon atoms respectively) and a few diterpenoids (4 isoprene units and 20 carbon atoms) are the volatile terpenoids that have very high vapor pressure and can be released into the atmosphere (El Hadi et al. 2013).

2.2.3 Biosynthesis of Volatile Compounds

Volatile compounds are synthesized from isopentenyl diphosphate (IPP), a universal 5 carbon precursor, and dimethylallyl diphosphate (DMAPP), IPP's allylic isomer (El Hadi et al. 2013). These two precursors can be produced via two different pathways in plants, the deoxyxylulose phosphate pathway, also called the mevalonate independent pathway, and the mevalonate pathway (McGarvey and Croteau 1995). The mevalonate pathway (MVA pathway)

occurs in the cytosol and starts with the condensation of an acetyl coenzyme A unit to an active isoprene unit (IPP) (Newman and Chappell 1999). The mevalonate independent pathway (MEP pathway) occurs in the cell plastids and forms IPP and DMAPP from pyruvate and glyceraldehyde 3-phosphate and uses methylerythritol phosphate (MEP), as the key intermediate (Lichtenthaler 1999).

After IPP and DMAPP are formed via these two pathways, IPP and DMAPP are combined in different ratios and acted upon by enzymes to create the precursors to the final volatile compound. A specific example of this is two molecules of IPP and one molecule of DMAPP being condensed in the cytosol, catalyzed by farnesyl pyrophosphate synthetase (FFPS) to produce farnesyl pyrophosphate, which is the natural precursor of sesquiterpenoids (McGarvey and Croteau 1995).

2.3 Phenolic Compounds and Volatile Compounds in Cranberries

2.3.1 Phenolic Compounds in Cranberries

Cranberries are a rich source of phenolic compounds containing flavonoid compounds such as flavan-3-ols/flavonols/anthocyanins, and phenolic acids such as hydroxybenzoic acid and hydroxycinnamic acids, along with their derivatives (Sanchez-Patan et al. 2012). The total phenolic makeup of cranberries contains approximately 20-25 individual phenolic compounds (Wang and Zuo 2011). The exact number of different individual phenolic compounds in cranberries vary in the literature, but hydroxybenzoic acid/ hydroxybenzoic acid derivatives, quercetin, and myricetin have been found by multiple studies to be the most prevalent phenolic compounds, and *o*-hydroxcinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acids, and epicatechin are present in moderate amounts (Wang and Zuo 2011) (Nowak et al. 2016). Fresh cranberries have also been found to have higher total phenolic levels (12.4 mg/g) than products

like cranberry juice (9.1 mg/g) and cranberry sauces (11.1 mg/g) (Want and Zuo 2011). This has posed as an important factor to consider for studies involving the effects of intake of cranberry phenolic compounds because fresh cranberries have an astringent and sour taste.

2.3.2 Volatile Compounds in Cranberries

Cranberry volatiles constitute several different types of compounds including aromatic compounds, terpenes, alcohols, and aldehydes (Croteau and Fagerson 1968). The aromatic compounds (benzaldehyde/benzyl/benzoate esters) and terpenes (α-terpineol) appear to be the major contributors to cranberry aroma, contributing to 40% and 17% of the total volatile fraction respectively in the study by Croteau and Fagerson (1968). Croteau and Fagerson also found that the terpene, α-terpineol, was the most abundant cranberry volatile, individually making up 13.6% of the total volatile fraction (1968). The study by Hirvi et al. (1981) mirrored these results, reporting aromatic compounds and terpenes making up 34% and 19% respectively, and α-terpineol individually making up 10% of the total volatile fraction. A third study by Anjou and Von Sydow (1967) found the aromatic compounds and terpenes made up 31% and 40% respectively and individual α-terpineol 23.7% of the total volatile fraction. The percentages of individual volatiles in cranberries varies in the literature, but all the literature concludes that aromatic compounds and terpenes contribute the largest amounts to the total volatile fraction, and α-terpineol is individually the most abundant volatile in cranberries.

2.4 Cranberry Consumption and Cranberry Health Benefits

2.4.1 Cranberry Consumption

According to the most recent report published by the United States Department of Agriculture, National Agriculture Statistics Service (NASS), the total amount of fresh cranberries consumed per person, per year, is 0.08 pounds (USDA, National Agriculture

Statistics Service 2014). Only these very small amounts of fresh cranberries are consumed likely due to the elevated hydroxybenzoic acid and proanthocyanin contents, which gives cranberries a very strong and astringent taste making them unacceptable to most palates in large amounts (Nile and Park 2014). Cranberry products, such as dried cranberries, cranberry juices, and cranberry sauces, even though they have lower total levels of bioactive compounds, are much more practical ways to consume cranberries due to addition of sugar to the products.

2.4.2 Cranberry Health Benefits

Several health benefits have been attributed to cranberry consumption including prevention of urinary tract infections, disruption of oral pathogen virulence and biofilm formation, modulation of the inflammatory response, and promotion of cardiovascular health (Pappas and Schaich 2009). Cranberries have also shown potential inhibiting effects on degenerative diseases including Alzheimer's and cancer (Pappas and Schaich 2009).

Prevention of urinary tract infections is the most extensively researched health benefit of cranberries. Urinary tract infections, in 75-95% of cases, are caused by the Escherichia coli pathogen adhering to the epithelial cells of the urinary tract, and the high acid content of cranberries prevents this adhesion to prevent infection (Zafrifri et al. 1989). Although it is now well accepted that cranberries aid in the prevention of urinary tract infections, the specific compounds in the cranberries that yield these protective properties are still up for debate.

Cranberries have been associated with dental health by reducing bacteria in the mouth and preventing plaque biofilm formation. The two main bacteria in the mouth responsible for dental caries are *Streptococcus mutans* and *Streptococcus sobrinus.* When these bacteria co-aggregate, they form biofilms and release acids that decay the teeth. Weiss et al. (1998) found that cranberry juice inhibited the co-aggregation of these bacteria in 58% of samples tested.

Other studies later confirmed that cranberry juice inhibited co-aggregation and biofilm formation, associating them with possible oral health benefits, but only if the cranberry juice was not supplemented with sugar (Bodet et al. 2006) (Wiess et al. 2002) (Steinberg et al. 2005). The *in vivo* evidence for cranberries' contribution to oral health is limited, but toothpastes or mouthwashes supplemented with cranberry phytochemicals appear to be promising.

Recent research also shows potential for cranberries to inhibit cancer cell growth. In a study by Seeram et al. (2006) cranberry extracts showed the ability to inhibit cancer cell growth, with higher concentrations of the extracts having a higher inhibitory effect in all of the tumor cell lines tested, which included oral, breast, colon, and prostate cancers. In 5 out of the 6 cell lines studied, the cranberry extract showed a significant inhibitory effect. Cranberry had the second lowest IC_{50} value for the MCF-breast cancer cell line and the fourth lowest IC_{50} value for the CAL-2-oral cancer cell line, indicating that the cranberry extract was a very potent growth inhibitor in these two cases (Seeram et al. 2006).

Consumption of cranberries is also suggested to play a role in cardiovascular health and neurological disease by decreasing the inflammatory response and increasing the antioxidant capacity in human plasma (Pappas and Schaich 2009).

2.5 Cranberry Anti-inflammatory Properties

2.5.1 Cranberry Phenolic Compounds Anti-inflammatory Activity

During inflammation, cells are faced with oxidative stress. Reactive oxygen species (ROS) are produced and considered one of the most potent stimuli for inflammation because they also stimulate the immune system monocytes/macrophages and increase the production of pro-inflammatory cytokines TNF- α, IL-8, IL-6 and IL-1β (Sultana and Saify 2012). Cranberry extracts have shown the ability to decrease the production of these reactive oxygen species and

pro-inflammatory cytokines. In the study by Mathison et al. (2014) subjects consumed acute doses of cranberry beverages. Blood was collected at 2-hr intervals from 0 to 8 hr and 24 hr after treatment and the biomarkers of antioxidant status were lower compared to starting values.

In the study by Bodet et al. (2006) macrophage cells were treated with a cranberry phenolic fraction before stimulation with lipopolysaccharide (LPS), another potent inducer of inflammation. After treatments with the cranberry fraction and LPS, the inflammatory markers TNF- α, IL-8, IL-6 and IL-1β were compared to positive and negative controls. The cranberry treated cells exhibited much lower levels, indicating that the cranberry fraction was a potent inhibitor of the pro-inflammatory responses induced by LPS.

2.5.2 Cranberry Volatile Compounds Anti-inflammatory Activity

There has been very little research conducted specifically on the anti-inflammatory properties of volatile compounds extracted from cranberries, hence the reason for this study, but total cranberry fractions have shown potent anti-inflammatory activity. Previously conducted research suggests that volatile compounds overall have anti-inflammatory and therapeutic potential due to their ability to suppress nuclear factor κB, a factor that controls transcription of DNA to produce the pro-inflammatory cytokines (Salminen et al. 2008). Previous research also indicates that individual volatile terpenoids in cranberries can act as possible anti-inflammatory agents. α-terpineol, which is by far the most abundant individual volatile compound in cranberries, has already shown the ability to inhibit the nuclear factor κB pathway (Hassan et al. 2010), thus suggesting it and possibly other individual volatile terpenoids in cranberries could play a role in the berries' anti-inflammatory action.

Chapter 3: Extraction, Quantification, Identification, and Free Radical-Scavenging Activity of Cranberry Phenolic Compounds

3.1 Introduction

In the literature regarding cranberries, the cranberry phenolic compounds have been suggested to be responsible for the majority of the berries' beneficial health properties, including their anti-inflammatory activity. This study took a different approach, suspecting other compounds may also contribute to cranberries' anti-inflammatory effect. The hypothesis of this study was that the volatile compounds, and not just the previously hypothesized phenolic compounds, also have anti-inflammatory capabilities when tested *in vitro* on RAW 264.7 cells. For this study, it was necessary to evaluate the cranberry phenolic extract for its *in vitro* anti-inflammatory effect on RAW 264.7 cells to be able to compare its effect to the effect of the volatile extract. At the time of this study, the phenolic compounds have been deemed to be the major bioactive source of cranberry's anti-inflammatory properties (La et al. 2010).

Most methods recommend using a mixture of organic solvents and polar solvents to extract phenolic compounds, especially for berries high in anthocyanin content like a cranberry (La et al. 2010). These recommendations were followed and used to extract the phenolic compounds for this study and are explained in section 3.3.1.

3.2 Materials

Cranberries (Stahlbush Island Farm brand) were purchased from Harps Foods, Fayetteville, AR. Stahlbush Island Farm is located on 5000 acres of land in the Willamette Valley of Oregon. The cranberries farmed there are left in the field until ripe, and frozen immediately after harvest to seal in flavor and freshness. After purchase from Harps Foods, the

cranberries were stored unopened and frozen at -20° C until used for experimentation to avoid any degradation of the phenolic compounds.

3.3 Methods

3.3.1 Extraction of Phenolic Compounds

Cranberries were thawed to room temperature and rinsed with running water to remove any dirt/debris. Three separate phenolic extracts were prepared from fresh cranberries. Approximately 100 g of the rinsed cranberries were homogenized with 250 mL methanol, water, and formic acid using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corporation, Mason, OH) for approximately 60 sec. The homogenate was then vacuum filtered through Miracloth (CalBioche, LaJolla, CA) and the resulting filtrate was collected. The resulting residue was homogenized using the Euro Turrax Tissuemizer with 250 mL of acetic acid, water and acetone for approximately 60 sec. The residue homogenate was vacuum filtered through Miracloth and combined with the previously collected filtrate. That resulting residue was homogenized, and vacuum filtered the same as the previous residue. The final residue and Miracloth were rinsed with both solvents, methanol/water/formic acid and acetic acid/water/acetone to collect any remaining cranberry compounds and added to the final filtrate. The final filtrate was placed in a Buchi Rotary Evaporator R-114 (Buchi, Flawil, Switzerland) set at 40°C to evaporate all of the solvents leaving only the phenolic extract. Next, the phenolic extract was centrifuged for 5 min at 10,000 rpm and supernatant collected. The extract was then loaded onto a Sep-Pak® C_{18} column cartridge (Waters Corporation, Milford, MA) and eluted with 70-100% ethanol until color was no longer visible in the cartridges. The samples were passed through 0.45 μm filters (Whatman) before HPLC analysis.

3.3.2 High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds

The individual phenolic compounds from the cranberry extract were separated by HPLC on a 250 X 4.60 mm Symmetry 5 μm C_{18} column (Waters Corp, Milford, MA). The resulting peaks were analyzed at 320, 360, and 510 nm using a Waters Model 996 photodiode array detector (Waters Corp, Milford, MA). Each sample injection volume was 100 μL. Solvent A was the mobile phase, water acidified with phosphoric acid to pH 2.6 and methanol was used as solvent B. The flow rate was 1.33 mL/min, and a gradient of the solvents was used to get the best separation of the compounds, starting with 88% A from 0-10 minutes, 85% A from 10-26 minutes, 40% A from 26-55 minutes, 30% A from 55-70 minutes, and 88% A from 70-85 minutes. Detection wavelengths of 320, 360, and 510 nm was used to monitor hydroxycinnamic acids, flavonols and anthocyanins, respectively. Individual anthocyanin monoglucosides and acylated anthocyanin derivatives were quantified as Cyd (cyanidin), Pnd (peonidin), and Mvd (malvidin) glucoside equivalents using external calibration curves of a mix of the three anthocyanin glucosides. Anthocyanins were quantified as a mixture of these glucosides because they are naturally occurring common anthocyanidins found in nature. Hydroxycinnamic acids were quantified as chlorogenic acid equivalents using external calibration curves of chlorogenic acid and flavonols were quantified as rutin equivalents using external calibration curves of rutin. Results are expressed as mg of anthocyanin-3-glucoside equivalents, chlorogenic acid equivalents, and rutin equivalents per kg of fresh weight.

3.3.3 Total Phenolic Content

The total phenolic content of the phenolic extract was measured using the Folin-Ciocalteu assay. First, 0.5 mL of 0.2 N Folin-Ciocalteu reagent was mixed with 0.1 mL of the cranberry phenolic extract. Next, 0.4 mL of 7.5% $NaHCO_3$ was added and mixed into the solution. The

solution was then placed into a 48-well plate in triplicate and let sit in the dark for 2 hr at room temperature. After the 2 hr, the absorbance of each sample was read at 760 nm. Total phenolic quantification was determined by comparing the absorbances of the samples to the absorbances of known gallic acid equivalents (GAE) at 100, 50, 25, 12.5, 6.25, and 3.125 mg per kg, which were prepared in the same way as the samples. Results are expressed as averages of each triplicate, with units of mg of GAE per kg fresh weight.

3.3.4 DMAC (4-dimethylaminocinnamaldehyde) Total Procyanidins Assay

Total procyanidins present in the phenolic extract was measured using the DMAC assay following the methods of Payne et al. (2010). A solution of 3 mL of HCl in 27 mL alcohol was prepared and then 0.03 g of DMAC was added to the solution (now be referred to as DMAC solution). Aliquots (50 μL) of blanks, standards, and extracts were prepared. 250 μL of DMAC solution was added to all prepared blanks, standards, extracts. Plate was read immediately at 640 nm. Catechin was used as the standard (2, 4, 8, 16, 32, and 64 mg/kg) with results expressed as mg of catechin equivalents per kg fresh weight.

3.3.5 DPPH (2,2-diphenyl-1-picrylhydrazyl) Total Antioxidant Capacity Assay of Phenolic Extract

Total antioxidant capacity of the phenolic extract was measured using a modified DPPH method published by Akkari et al. (2016). 1.4 mL of a solution of DPPH in methanol was added to 0.1 mL of the phenolic extract at 10x, 20x, 40x, 80x, and 160x dilutions. The combined solution sat in the dark for 30 minutes, then absorbance on a spectrophotometer at 517 nm was measured. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as the standard (50, 100, 200, 400, 800 μM) with results expressed as μM of Trolox per kg of sample.

3.4 Results

3.4.1 HPLC

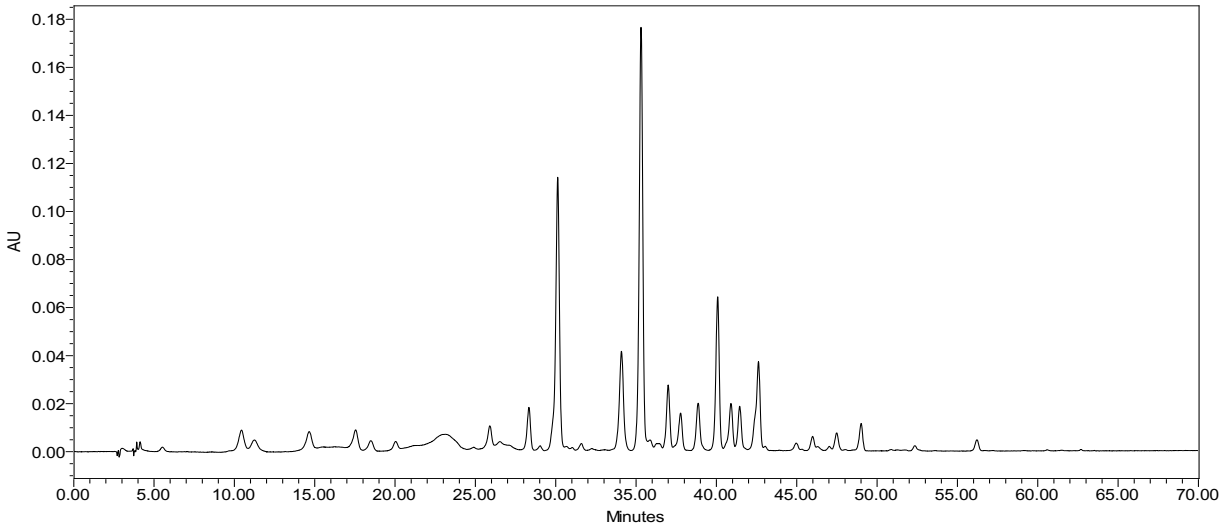


Figure 3.1 HPLC chromatogram at 360 nm measuring for flavonols

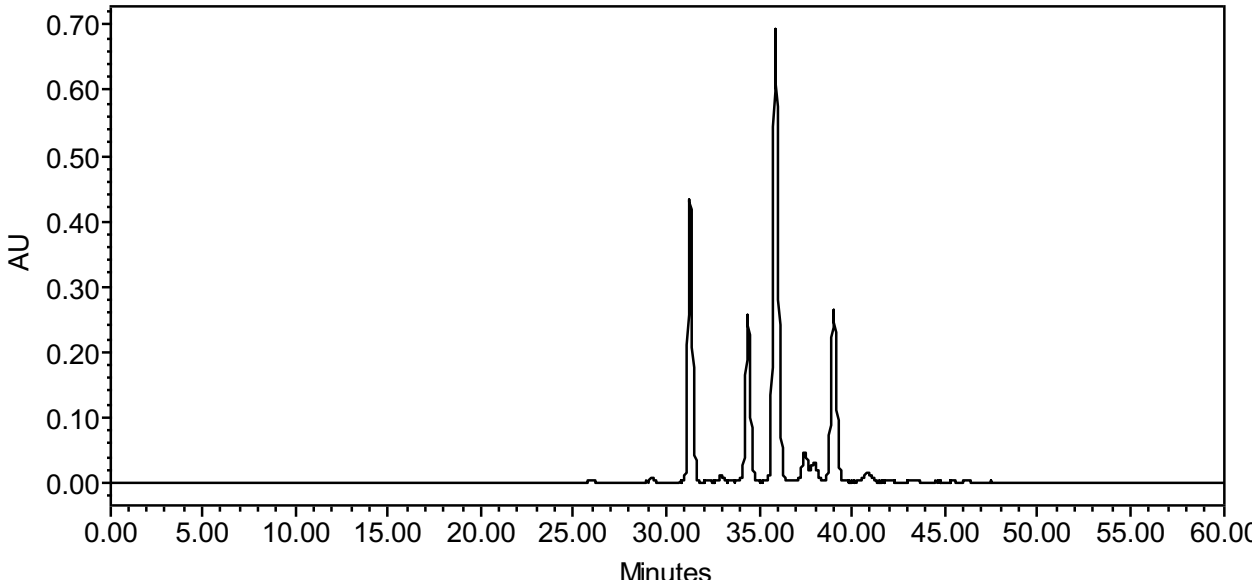


Figure 3.2 HPLC chromatogram at 510 nm measuring for anthocyanins

Table 3.1 Composition of anthocyanins, flavonols, and hydroxycinnamic acids in cranberry extract

Anthocyanins (mg ACY-3-Glu equiv. /kg FW)		**Flavonols (mg Rutin equiv./kg FW)**		**Hydroxycinnamic acids (mg Chlorogenic acid equiv./kg FW)**	
Cyd-3-gal	148.0±1.0	Myr-3-pentoside	6.7±0.0	Chlorogenic acid	14.7±0.0
		Myr-3-pentoside	0.7±0.0		
Cyd-3-glu	2.7±0.0	Quer-3-xylo	3.4±0.0		
Cyd-3-arab	93.1±1.0	Quer-3-arbinopyranoside	9.2±0.0		
Mal-3-gal	8.0±2.0	Myr-3-gal	17.4±0.0		
Peo-3-gal	489.8±4.0	Quer-3-arabinofuranoside	3.4±0.0		
		Quer-3-gal	25.5±0.0		
Peo-3-arab	195.0±2.0	Quer-3-glu	2.7±0.0		
		Quer-3-rham	2.7±0.0		
		Isorham-3-hexoside	2.7±0.0		
		Isorham-3-pentoside	5.4±0.1		
		Isorham-3-pentoside	0.7±0.0		
		Isorham-3-pentoside	1.3±0.0		
		Isorham-3-pentoside	1.3±0.0		
		Quer-3-benzoyl-gal	0.7±0.0		
Total Anthocyanins	**936.6±8.0**	**Total Flavonols**	**84.4±1.0**	**Total Hydroxycinnamic acids**	**14.7±0.0**

Anthocyanins were quantified using an external calibration curve of a mix of the three predominant anthocyanin glucosides found in cranberries; Cyd (cyanidin), Pnd (peonidin), and Mvd (malvidin) glucoside equivalents per kg of fresh weight. Flavonols were quantified using an external calibration curve of rutin and are expressed as mg of rutin equivalents per kg of fresh weight. Hydroxycinnamic acids were quantified using a chlorogenic acid external calibration curve and are expressed as mg of chlorogenic acid equivalents per kg of fresh weight.

3.4.2 Total Phenolic Content Using Folin-Ciocalteu Assay

Table 3.2 Total phenolic content of cranberry extract

	abs 760 nm	extract dilution	assay dilution	**Total Phenolics**
Cranberry phenolics	0.3593	0.67	50	1240
Cranberry phenolics	0.3700	0.67	50	1277
Cranberry phenolics	0.3760	0.67	50	1297
				Average 1271

Total Phenolics are expressed as the triplicate average with units of mg of gallic acid equivalents (GAE) per kg fresh weight.

3.4.3 DMAC (4-dimethylaminocinnamaldehyde) Total Procyanidins Assay

Table 3.3 Summary statistics of DMAC analysis of phenolic extract

Mean	323.6
Std Dev	12.7
Std Err Mean	3.0
Upper 95% Mean	329.9
Lower 95% Mean	317.4
N	3
Total procyanidins	**323.6 mg/kg+/-2.9 SEM**

3.4.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical-Scavenging Assay of Phenolic Extract

Table 3.4 Summary statistics of DPPH analysis of phenolic extract.

Mean	4676.4
Std Dev	230.0
Std Err Mean	93.9
Upper 95% Mean	4917.7
Lower 95% Mean	4435.1
N	3

Total Antioxidant Capacity **4676.4 μM/kg+/-93.9**

3.5 Discussion

3.5.1 HPLC Analysis Discussion

HPLC analysis was used to determine the composition of the anthocyanins, flavonols, and hydroxycinnamic acids (a subcategory of phenolic acids) present in the phenolic extract. In this analysis, there were seven different anthocyanin compounds present, with total anthocyanins equaling 936.6 ± 8.0 mg ACY-3-glucoside equiv. per kg fresh weight, sixteen different flavonol compounds present, with total flavonols equaling 84.4 ± 1.0 mg rutin equiv. per kg fresh weight, and one hydroxycinnamic acid identified, with total hydroxycinnamic acids equaling 14.7 ± 0.0 mg chlorogenic acid equiv. per kg fresh weight (Table 3.1). It appears that the cranberry phenolic profile has been well mapped, because the literature is in agreeance with the results of this study. Although some studies reported results on dry weight basis instead of fresh weight, all studies reported the highest concentration of classes of compounds to be the proanthocyanidins and anthocyanins, at 133 g per kg dry weight and Wu et al. (2006) only looked specifically at total anthocyanin content and reported a value of 1400 ± 28.5 mg per kg of fresh weight, which is comparable to the 936.6 ± 0.8 mg per kg of fresh weight found in this study. One thing that did

seem to differ among the literature was the number of compounds identified in the cranberry phenolic fraction, found to be twenty-three in this study and reported at seventeen and eleven in others (Seeram et al. 2006, Gregoire et al. 2007). In the study that reported only eleven highly purified compounds by using HPLC analysis to separate flavonols, phenolic acids, and proanthocyanins of cranberries they identified the following; quercetin, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-arabinofuranoside, quercetin-3-rhamnoside, myricetin, myricetin-3-rhamnoside, epicatechin, epicatechin-(4β-8)-epicatechin, caffeic acid, and chlorogenic acid (Gregoire et al. 2007), and two of those were not identified in this study. Differences were likely due to differences in analytical procedures. Three different wavelengths were monitored to identify the anthocyanins, flavonols, and hydroxycinnamic acids in this study, whereas Gregoire et al. (2007) did not report the wavelength(s) used to identify the compounds. The differences among the two studies could also be due to analysis of different cranberry varieties.

3.5.2 Total Phenolics by Folin-Ciocalteu Discussion

The total phenolic content of the cranberry extract used in this study was 1271 mg GAE per kg fresh weight, shown in Table 3.2. Upon comparison to other studies analyzing cranberry extract for total phenolics, results seem to vary. The 1271 mg GAE per kg fresh weight found in this study is low compared to the 1627 mg GAE per kg of fresh weight reported by Nowak et al. (2016). Results of this study also seem low compared to the study by Abeywickrama et al. (2016), who reported the total phenolics of market-mature cranberries to be ~6100 mg GAE per 100 g dry weight. Due to the different nature of the method (fresh weight vs. dry weight) it is expected that the dry weight would be much higher because of concentration of the phenolic compounds during the drying process. The cranberries used in these studies could also be

different varieties or cultivated in different methods resulting in the differing values for total phenolic content.

3.5.3 DMAC (4-dimethylaminocinnamaldehyde) Total Procyanidins Discussion

Total procyanidins of the cranberry extract used in this study were found to be 324 mg/kg. The literature reports greatly varying values, Wallace and Giusti (2010) evaluated eight different cranberry samples and found total procyanidins varying from 780 to 22,450 mg per kg. Another study found total procyanidins of cranberry extract to be 5 mg per kg, total procyanidins of whole cranberries to be 17 mg per kg, and total procyanidins of two cranberry juices to be 223 and 216 mg per kg (Prior et al. 2001). The large variation in total procyanidins reported in the literature and found in this study could be due to different starting cranberry products (different varieties of fresh cranberries, freeze dried cranberries, cranberry juices ect.) and different extraction and analytical methods used for quantification.

3.5.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) Total Antioxidant Capacity Discussion

Total antioxidant capacity of the phenolic extract used in this study was found to be 4,676 μM Trolox per kg. In the literature, using the oxygen radical absorbance capacity assay (OREC) cranberry anti-oxidant capacity has been reported as high as 95,840 μM Trolox equivalent per kg and ranked number one among fifty fruits and vegetables evaluated (Floegel et al. 2011), however different methods to evaluate total antioxidant capacity yield different results. The DPPH method in Floegel et al. (2011) study reported antioxidant capacity at 868 mg vitamin C equivalents per kg fresh weight, which is high compared to the other fruits and vegetables tested, but hard to directly compare to this study as Trolox is a vitamin E derivative. Borowska et al. (2009) reported the antioxidant capacity of wild cranberry to be 36,900 μM Trolox per kg and Wang and Stretch (2001) reported 8,200 – 10,100 μM Trolox per kg in their study. The large

variation of antioxidant capacity found in this study and in the literature is likely due to differing cranberry varieties, different cultivar techniques, different cranberry starting products, and different extraction and quantification methods.

Chapter 4: Extraction, Quantification, Identification and Free Radical-Scavenging Activity of Cranberry Volatile Compounds

4.1 Introduction

To discover whether the volatile compounds in cranberries also have an anti-inflammatory effect, the volatile compounds from the cranberries needed to be extracted for *in vitro* experimentation on the RAW 264.7 cells. The volatile extract also was further analyzed using Gas Chromatography – Mass Spectrometry (GC-MS) to determine which individual volatile compounds were present. Due to the instability of volatile compounds at high temperatures, vacuum distillation followed by rapid cooling, to avoid any degradation, was used for isolation of the individual volatiles (Belitz et al. 2009).

4.2 Materials

Cranberries (Stahlbush Island Farm brand) were purchased from Harps Foods, Fayetteville, AR. Stahlbush Island Farm is located on 5000 acres of land in the Willamette Valley of Oregon. The cranberries farmed there are left in the field until ripe, and frozen immediately after harvest to seal in flavor and freshness. After purchase from Harps Foods, the cranberries were stored unopened and frozen at -20° C until used for experimentation to avoid any degradation of the volatile compounds.

4.3 Methods

4.3.1 Extraction of Volatile Compounds

Cranberries were thawed to room temperature and rinsed with running water to remove dirt and debris. Three separate volatile extracts were prepared from fresh cranberries. Volatiles were obtained by combining 300 g of berries, 300 mL deionized water, and 100 g NaCl and blending for one min in a Waring blender. The homogenate was vacuum distilled at 28 in. Hg, 50°C water bath, 0ºC condenser for 30 min using a Buchi rotary evaporator (Buchi, Flawil, Switzerland). The first 200 mL was collected in a flask contained in an ice water bath. Additional ice packs were strapped to the condenser to aid in condensation and collection of volatiles. The final collected cranberry extract was put in a glass jar, sealed, and immediately stored in the freezer at -20 ºC until used for experimentation.

4.3.2 Solid Phase Micro-extraction (SPME) of Volatiles

An 85μm, CAR/PDMS, Stableflex, 24 Ga, Manual Supelco (Bellefonte, PA) SPME fiber was used in this study. Vials containing 4 mL of volatile extract were placed on a stir plate with temperature set at 65° C with the SPME fiber inserted into the headspace above the sample. Adsorption was timed for 30 min. Samples of volatiles (100 μL) were placed into 1 mL vials. After preheating for 5 min at 40ºC, headspace volatiles were collected by SPME for 20 min at 60ºC using a DVB/CAR/PDMS fiber (Supleco Inc., Bellafonte, PA).

4.3.3 GC Quantification of Volatiles

Volatiles adsorbed to the SPME fibers were desorbed at 270 °C for 2 min in the injection port of a Varian 3800 GC (Agilent Technologies, Santa Clara, CA) equipped with a HP-5 (5% phenyl-methylpolysiloxane) column (30 m X 250 um X 1 um) (Agilent Technologies, Santa Clara, CA). The FID detector was at 280°C. GC runs were 29 min, and the fiber remained in the

injection port for 10 min after each run. The injection port was operated in splitless mode with a constant He flow of 25 psi. The initial oven temperature was 25° C, held for 4 min, ramped up at 12 °C/ min to 289° C, and held at for 3 min. Volatiles were quantified as heptanal, octanal, alpha phellandrene, d-limonene, limonene, ocimene, nonanal, terpin-4-ol, alpha terpineol, beta caryophyllene, and alpha caryophyllene.

4.3.4 GC-MS Identification of Volatiles

SPME-collected volatiles were analyzed by GC-MS using a Hewlett-Packard HP 5890 series gas chromatograph equipped with a mass selective detector (MSD) and a HP-5 capillary column (Agilent, 30 m x 0.25 mm, film thickness 1 μm). Working conditions included: injector temperature 270°C; MSD interface temperature 280°C; oven temperature programmed from -10°C (1 min) to 280°C at 12°C/min; carrier gas (He) at a flow rate of 0.78 mL/min; injection port operated in splitless mode. MSD acquisition parameters included full scan mode, scan range 20-300 *m/z* and scan speed 3.2 scans/s. Volatiles were identified by comparing their mass spectra with the spectral library (Wiley7NIST0.5), literature data, and alkane retention indices C5-C20.

4.3.5 Total Phenolic Content

The total phenolic content of the volatile extract was measured using the Folin-Ciocalteu assay. First, 0.5 mL of 0.2 N Folin-Ciocalteu reagent was mixed with 0.1 mL of the cranberry volatile extract. Next, 0.4 mL of 7.5% $NaHCO_3$ was added and mixed into the solution. The solution was then placed into a 48-well plate in triplicate and let sit in the dark for 2 hr at room temperature. After the 2 hr, the absorbance of each sample was read at 760 nm. Total phenolic quantification was determined by comparing the absorbances of the samples to the absorbances of known gallic acid equivalents (GAE), which were prepared in the same way as the samples.

Results are expressed as averages of each triplicate, with units of mg of GAE per 100 g fresh weight.

4.3.6 DPPH (2,2-diphenyl-1-picrylhydrazyl) Total Antioxidant Capacity Assay of Volatile Extract

Total antioxidant capacity of the phenolic extract was measured using a modified DPPH method published by Akkari et al. (2016). 1.4 mL of a solution of DPPH in methanol was added to 0.1 mL of the phenolic extract at 10x, 20x, 40x, 80x, and 160x dilutions. The combined solution sat in the dark for 30 minutes, then absorbance on a spectrophotometer at 517 nm was measured. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as the standard (50, 100, 200, 400, 600, 800 μM) with results expressed as μM of Trolox per Kg of sample.

4.4 Results

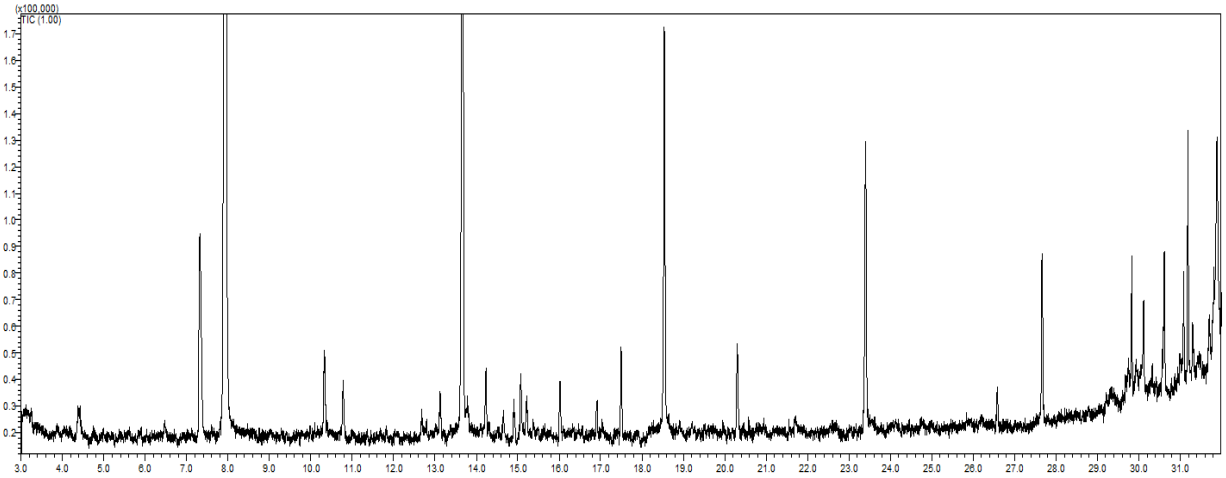


Figure 4.1 GC-MS Chromatogram of cranberry volatiles.

Table 4.1 Composition of cranberry volatiles using GC-MS

Retention Time[1] (min)	Volatile Compound	Concentration (ppb)
4.22	1-Butanol	18.19
8.12	1-Pentanol	24.58
9.1	Hexanol	24.73
11.97	Heptanal	6.78
13.65	1-heptanal	14.64
13.45	Benzaldehyde	17.94
13.87	1-octen-3-ol	16.85
14.4	Octanal	10.87
14.57	Alpha phellandrene	27.17
14.98	D-limonene	22.66
15.06	Limonene	4.20
15.17	Eucalyptol	189.04
15.21	Ocimene	40.25
15.36	Ocimene	7.76
15.63	2-octanal	110.26
15.88	Linalool oxide	224.42
16.35	Linalool	41.32
16.5	Nonanal	15.46
16.58	Nd	46.73
18.09	Terpin-4-ol	74.36
18.45	Alpha terpineol	2320.55
19.46	Carvone	45.85
19.61	Trans-2-decanal	134.20
22.59	Beta caryophyllene	54.68
23.21	Alpha caryophellene	58.00
	Total Volatiles	**3551.5**

Nd, not determined.
[1]Retention times correspond to retention times in Figure 4.2.

The results of the Folin-Ciocalteu assay measuring for total phenolics of the cranberry volatile extract was zero, as expected.

Table 4.2 Summary statistics of DPPH analysis of volatile extract.

Mean	40.2
Std Dev	1.8
Std Err Mean	0.72
Upper 95% Mean	42.0
Lower 95% Mean	38.3
N	6

Total Antioxidant Capacity **40.2 μM/Kg+/-0.72 SEM**

4.5 Discussion

The chromatogram from Figures 4.1 contain a large number of peaks, which displays the complex aromatic composition of cranberries. 25 individual volatile compounds in the cranberry volatile extract used for this study were identified using Mass Spectrometry analysis and are listed in Table 4.1. This number of compounds is lower than other reported studies, with a comprehensive literature review reporting values for food volatiles compiling the data from six cranberry studies reporting a total of 115 volatile compounds previously identified in cranberries (De Vincenzi et al. 1989). In one of the studies reviewed that used American cranberry, 42 compounds were identified, which is closer to 25 compounds identified in this study (Croteau and Fagerson 1968). The data from the previous studies should be compared to the current study with discretion, taking into account that some of the papers reviewed used cranberry juice, European cranberries, and cranberry press cake, which was denoted as the berry residue remaining after the juice has been expressed, as the starting cranberry component for evaluation, whereas the cranberries in this study are American cranberries grown and harvested in Oregon (Anjou et al. 1967). Another thing possibly leading to these discrepancies with the literature is

that the extracts obtained from the starting cranberry components were prepared for evaluation in different manners, for example Croteau and Fagerson (1968) used a cold press technique and this study used vacuum distillation.

The Folin- Ciocalteu assay was used to measure total phenolic content of the volatiles and the values were zero, as expected. Table 4.2 show the results of the DPPH assay, measuring for total antioxidant capacity, with total antioxidant capacity of the volatile extract calculated as 40.2 uM/kg +/- 0.72. Results for both of these assays were very low as expected. These assays are typically not performed on volatile extracts, but they were in this study to keep consistency between the data collected from the phenolic extract and the volatile extract. The results demonstrate that the volatile fraction does not contain phenolics and the antioxidant activity of the volatile fraction is very low when compared with the phenolic fraction.

Chapter 5: *In Vitro* Anti-Inflammatory Effect of Cranberry Phenolic and Volatile Extracts on RAW 264.7 cells.

5.1 Introduction

To evaluate the anti-inflammatory capabilities of the cranberry phenolic and volatile extracts, an *in vitro* nitric oxide (NO) assay was performed. Nitric oxide assays are used to measure the total amount of nitrate/nitrite and are commonly used in experiments studying free radical scavenging, anti-cancer properties, anti-ageing properties, and anti-inflammatory properties (Kagoo and Chellathai 2014). In this case, NO levels were measured as an inflammatory marker. A set number of cells were treated with LPS (to stimulate inflammation and NO production), then treated with another substance and given time for that substance to have its' effect on the cells (level of NO produced). Then the NO levels produced by the treated

cells compared to non-treated (control) were measured using colorimetric analysis to assess the anti-inflammatory properties of the treatments.

One concern that had to be addressed in methodology of this study was the solubility of the volatile compounds in the aqueous media. Because of the lipophilic nature of volatile compounds, a surfactant was needed to ensure suspension of the volatile compounds in the volatile treatment media. In all dilutions of the volatile treatments, there was a final concentration of 0.02% tween 80, a non-ionic surfactant, to ensure that the volatiles dispersed throughout the experimental media and were available to treat the cells. Tween 80 was chosen because it has been previously shown to work in cell culture and to hold volatile compounds in suspension in cell culture media (O'Sullivan et al. 2004). Tween 80 was found to be non-cytotoxic to RAW 264.7 cells at the 0.02% concentration used in this study using the CellTiter 96® Aqueous One Solution Cell Proliferation (Inah Gu, MS Thesis).

RAW 264.7 cells, a mouse macrophage cell line from mouse blood, were chosen for the NO assay because the macrophage is a major cell type involved in inflammation, and because of the well-known anti-inflammatory properties of cranberries (Bodet et al. 2006).

5.2 Materials

Cranberry phenolic and volatile extracts, explained in chapters 3 and 4 respectively, were used as the treatments in cell culture prior to the NO assay. The RAW 264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum (FBS). The components for the DMEM media and all cell culture experimental reagents were purchased from Gibco® through

Life Technologies (Carlsbad, CA). The Griess Reagent System kit was purchased from Promega Corporation (Madison, WI).

5.3 Treatment Dosage of Cranberry Phenolic and Volatile Extracts

Phenolic and volatile extracts (reference chapters 3 and 4) were used as experimental treatments on RAW 264.7 cells before measuring the NO levels. The extracts were brought back to the original starting weight of the fresh cranberries so that they accurately reflect the natural concentrations of the phenolics and volatiles in a fresh cranberry. Due to the cells need for nutrient-rich media, the extracts could not be applied directly to the cells because of potential cell death. To keep the treatment dosage as high as possible, 2x, 4x, and 8x dilutions of the phenolic and volatile extracts were used. The prescribed 2x, 4x, and 8x dilutions of phenolic and volatile extracts were found to be non-toxic to the cells via the The CellTiter 96® AQueousOne Solution Cell Proliferation Assay in a preliminary study (Inah Gu, MS Thesis).

Treatment of the RAW 264.7 cells in this manner, with the same dilutions of phenolic and volatile extracts, rather than treatment with the same concentration of each extract, is justified by the fact that the concentrations of these compounds are not equivalent in a fresh cranberry. This study is representative of the amount of phenolics and volatiles that a person would obtain by eating fresh cranberries. Actual concentrations of treatment extracts are listed in Table 5.1.

Table 5.1 Concentrations of cranberry phenolic and volatile compounds applied as treatments to RAW 264.7 Cells

Treatment	**[1]Actual Concentration**	**2x dilution**	**4x dilution**	**8x dilution**
Volatile Fraction	3.6 ppm	1.80 ppm	0.90 ppm	0.45 ppm
Phenolic Fraction	1271.3 ppm	635.7 ppm	317.8 ppm	158.9 ppm

[1]Actual concentration refers to starting concentration in total extracts.

5.4 Methods

5.4.1 Cell Culture

RAW 264.7 mouse microphage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum (FBS). The cells were maintained in 75 cm^2 cell culture flasks and incubated at 37 °C in a 5% CO_2 environment. All NO assays were conducted between cell passage numbers 4-8. The components for the DMEM media and all experimental reagents were purchased from Gibco® through Life Technologies (Carlsbad, CA).

5.4.2 Griess Reagent System Assay (NO Assay)

Nitric oxide production was analyzed using the Griess Reagent System kit containing nitrite standard, N-1-napthylethylenediamine dihydrochloride (NED) solution, and sulfanilamide solution, which was purchased from Promega Corporation (Madison, WI). 100 μL of RAW 264.7 cells in enriched DMEM media were seeded in a 96 well plate (plate 1) and incubated at 37 °C and 5% CO_2 for 16 hr. After 16 hr, which allowed the cells to attach to bottom of the wells in the plate, the media was removed and treatment media containing a range of phenolic and volatile cranberry extracts was added, tween 80 was added at a concentration of 0.02% to the volatile treatment media. The treatment media was left on the cells for 1 hr, and then removed. Next, lipopolysaccharide (LPS) media, concentration 100 ng LPS per mL, was applied to the cells and left on for 24 hr. After 24 hr, the LPS media was removed and the nitric oxide levels were measured. To measure nitric oxide, a nitrite standard reference curve, with concentrations ranging from 0 to 100 μM, was prepared on a separate 96 well plate (plate 2). Samples on plate 1 were centrifuged for 1 min at 1000 rpm and the supernatants from the samples were added to

plate 2. Then, 50 µL of sulfanilamide solution was added to the standard reference curve and the samples on plate 2 and allowed to sit for 10 min protected from light. After ten min, 50 µL of NED solution was added to all wells on plate 2 and allowed to sit protected from light for 10 min. After 10 min, the absorbance was read on a plate reader at 540 nm. After the absorbance readings, corrections were made to account for background absorbance of the sample control media. The absorbance readings were converted into nitric oxide levels using the slope value from the nitric oxide standard curve.

This experiment was conducted as described above, with the cranberry treatments applied before the LPS, and the reverse, with the cranberry treatments applied after the LPS. For the reverse, the RAW 264.7 cells were seeded in the 96 well plate and allowed to sit for 16 hr. After 16 hr the media was removed and the LPS media was applied. The LPS media was left on the cells for 24 hr. After 24 hr the cranberry phenolic and volatile treatment media containing the different concentrations was added, and a final concentration of 0.02% tween 80 was added to the volatile treatment media. After 1 hr with the cranberry treatment media, the plate was centrifuged, the supernatant transferred to a second plate, and the nitric oxide levels measured in the same way as described when treatments were applied before LPS.

5.5 Statistical Analysis

All statistical analysis was performed using JMP® Pro Ver. 14 Statistics Software. One-way analysis of variance for all responses for all treatment dilution combinations before and after by each treatment combination was ran in the Fit Y by X platform of JMP Pro Ver. 14. Multiple comparisons were performed using the Tukey HSD test.

Paired t-tests were used in the Matched Pair platform of JMP Pro Ver. 14 to compare the before and after LPS results for each treatment.

5.6 Results

5.6.1 Phenolic and Volatile Extract Treatments after Applying LPS

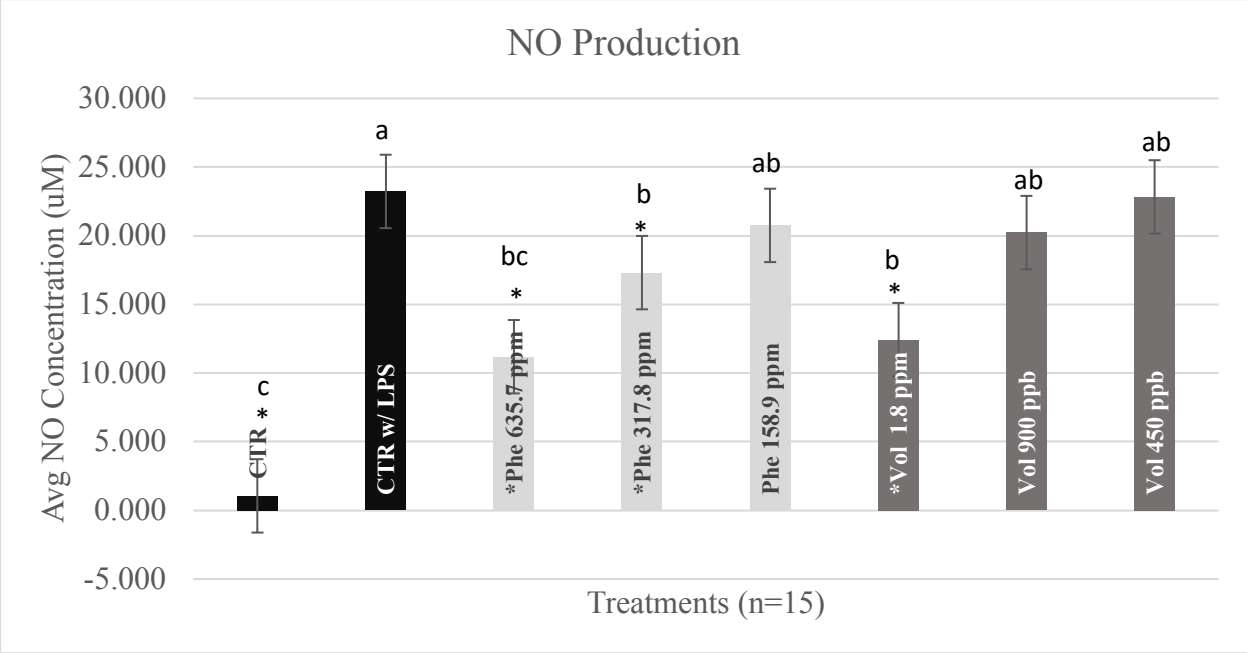


Figure 5.1 Average NO concentration produced by RAW 264.7 cells treated with phenolic and volatile compounds after applying LPS. * indicates a significant difference from the positive control (CTR w/ LPS). Levels not connected by the same letter are significantly different.

Table 5.2 Means comparisons of all pairs treatment after LPS using Tukey-Kramer HSD Confidence Quantile.

	Control-0x+	**Volatile-8x**	**Phenolic-8x**	**Volatile-4x**	**Phenolic-4x**	**Volatile-2x**	**Phenolic-2x**	**Control-0x-**
Control-0x+	-6.6141	-3.3605	-3.2148	-1.7156	0.0595	0.7510	2.3771	8.3440
Volatile-450 ppb	-3.3605	-6.6141	-6.4684	-4.9692	-3.1941	-2.5026	-0.8765	5.0904
Phenolic-158.9 ppm	-3.2148	-6.4684	-6.6141	-5.1150	-3.3398	-2.6483	-1.0222	4.9446
Volatile-900 ppb	-1.7156	-4.9692	-5.1150	-6.6141	-4.8390	-4.1474	-2.5214	3.4455
Phenolic-317.8 ppm	0.0595	-3.1941	-3.3398	-4.8390	-6.6141	-5.9226	-4.2965	1.6704
Volatile-900 ppb	0.7510	-2.5026	-2.6483	-4.1474	-5.9226	-6.6141	-4.9880	0.9788
Phenolic-635.7 ppm	2.3771	-0.8765	-1.0222	-2.5214	-4.2965	-4.9880	-6.6141	-0.6472
Control-0x-	8.3440	5.0904	4.9446	3.4455	1.6704	0.9788	-0.6472	-6.6141

Positive values show pairs of means that are statistically significant.

5.6.2 Phenolic and Volatile Extract Treatments before Applying LPS

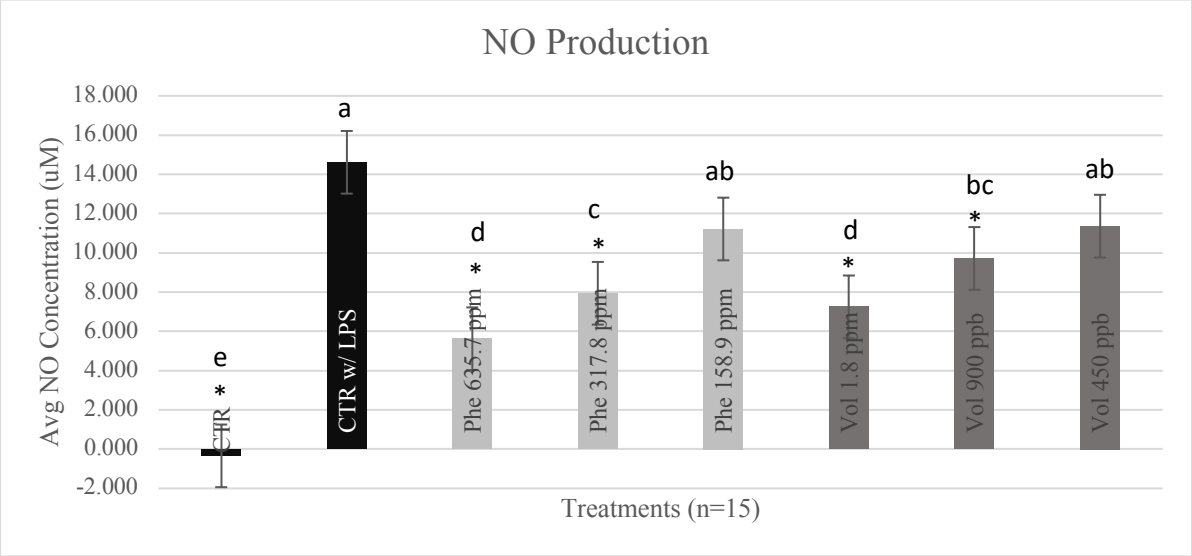


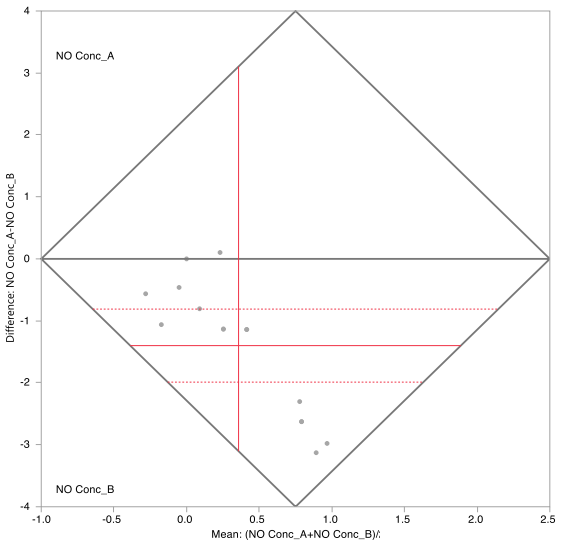
Figure 5.2 Average NO concentration produced by RAW 264.7 cells treated with phenolic and volatile extracts before applying LPS. * indicates a significant difference from the positive control (CTR w/ LPS). Levels not connected by the same letter are significantly different.

Table 5.3 Means comparisons of all pairs treatment before LPS using Tukey-Kramer HSD Confidence Quantile

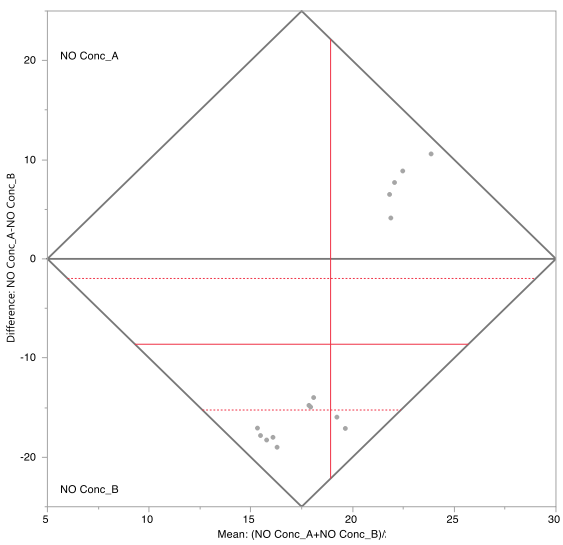
	Control-0x+	**Volatile-8x**	**Phenolic-8x**	**Volatile-4x**	**Phenolic-4x**	**Volatile-2x**	**Phenolic-2x**	**Control-0x-**
Control-0x+	-2.934	-2.534	-0.459	0.064	2.978	7.858	9.101	19.235
Volatile-450 ppb	-2.534	-2.934	-0.859	-0.336	2.579	7.458	8.701	18.835
Phenolic-158.9 ppm	-0.459	-0.859	-2.934	-2.411	0.503	5.383	6.626	16.760
Volatile-900 ppb	0.064	-0.336	-2.411	-2.934	-0.019	4.860	6.103	16.237
Phenolic-317.8 ppm	2.978	2.579	0.503	-0.019	-2.934	1.946	3.188	13.322
Volatile-1.8 ppm	7.858	7.458	5.383	4.860	1.946	-2.934	-1.691	8.443
Phenolic-635.7 ppm	9.101	8.701	6.626	6.103	3.188	-1.691	-2.934	7.200
Control-0x-	19.235	18.835	16.760	16.237	13.322	8.443	7.200	-2.934

Positive values show pairs of means that are statistically significant.

5.6.3 Analysis of Treating After vs. Treating Before Applying LPS

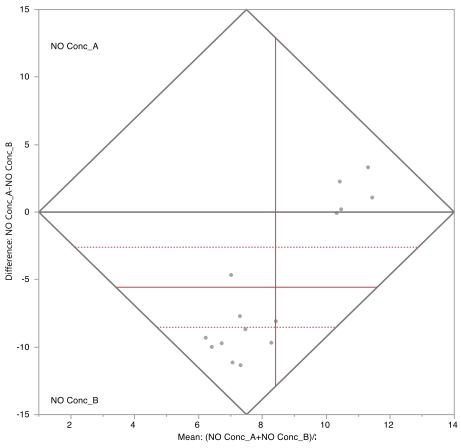


Control-0x-

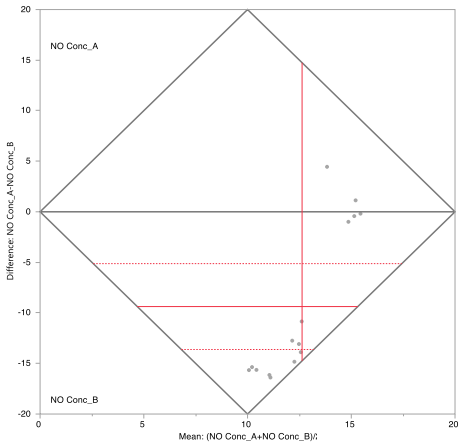


Control-0x+

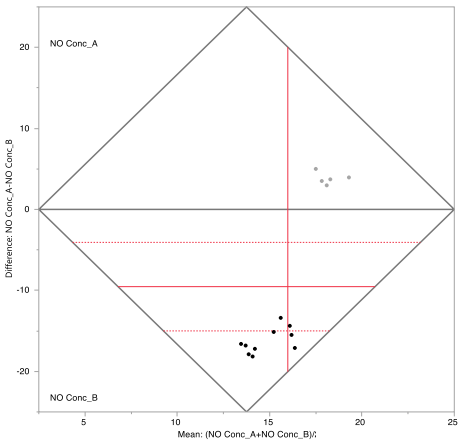
Figure 5.3 Comparison of NO levels of controls in experiments before and after LPS. NO Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.



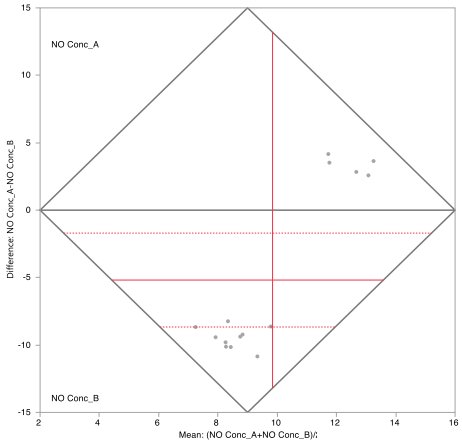
Phenolic 635.7 ppm



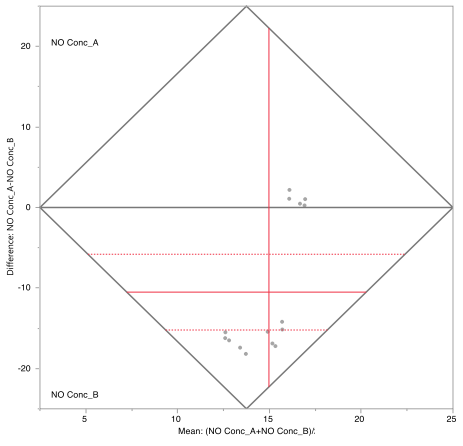
Phenolic 317.8 ppm



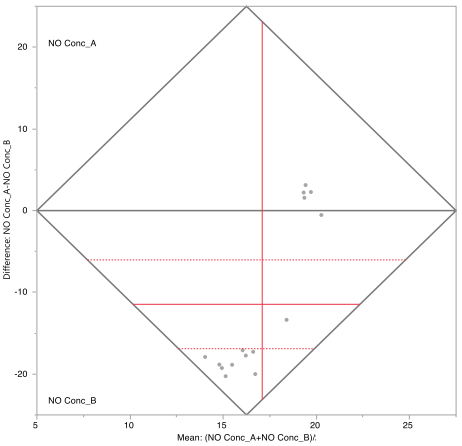
Phenolic 158.9 ppm



Volatile 1.8 ppm



Volatile 900 ppb



Volatile 450 ppb

Figure 5.4 Comparison of NO levels treating with phenolic and volatile extracts before and after LPS. Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.

5.7 Discussion

Upon statistical analysis of the data, the results of this study supported the proposed hypothesis that the volatile compounds contributed along with the phenolic compounds in the anti-inflammatory effect of cranberries. First, the results of application of the treatments after applying LPS will be discussed. Figure 5.1 along with the data listed in Table 5.2 show that application of cranberry phenolic and volatile extract treatments after applying LPS decreased NO levels compared to the positive control. The phenolic 635.7 ppm and 317.8 ppm treatments and the volatile 1.8 ppm treatment significantly lowered the amount of NO produced compared to the positive control. A relevant dose response is also shown in the results. That is as the dilution factor of the treatment extracts increased, the significance of the NO levels compared to the positive control decreased, showing that the more concentrated treatment extracts worked better to eliminate the pre-existing NO induced by LPS. Interestingly, the results of the phenolic 635.7 ppm and volatile 1.8 ppm treatment extracts were not significantly different from one another, but were both significantly different from the positive control, indicating they had the same treatment effect on lowering pre-existing NO. This is important to note because although both extracts were prepared at a 2x dilution of the starting concentration in a cranberry, the 2x dilution of the volatile extract was 353x less than the concentration of the 2x dilution of the phenolic extract. So even at this extremely low concentration the volatile extract at 1.8 ppm worked just as well as the phenolic extract at 635.7 ppm at treating pre-existing NO. Also, the phenolic extract treatment at 635.7 ppm shared a letter with the negative control, the cells without any exposure to LPS, indicating that the NO levels of the phenolic 635.7 ppm extract treatment showed no significant difference from the cells that had no inflammation induced. P-values of all treatments compared to the positive control are listed in the appendix, with the

significant values as follows: phenolic 635.7 ppm with a p-value of 0.0014, phenolic 317.8 ppm with a p-value of 0.0463, and volatile 1.8 ppm with a p-value of 0.0180.

The results of the treatment of the cells before applying LPS also supported the hypothesis that the volatile compounds contribute along with the phenolic compounds in the anti-inflammatory effect of cranberries. Figure 5.2 along with the data in Table 5.3 shows that application of cranberry phenolic and volatile extract treatments before applying LPS decreased NO levels compared to the positive control, demonstrating a preventative effect. Both phenolic and volatile extract treatments at 2x (635.7 ppm and 1.8 ppm respectively) and 4x dilutions (317.8 ppm and 900 ppb respectively) significantly lowered the amount of NO produced compared to the positive control. A relevant dose response is also shown in the results when the experiment was conducted in this manner. As the dilution factor of the treatment extracts increased, the significance of the NO levels compared to the positive control was reduced, showing that the more concentrated treatment extracts worked better to prevent the cells from producing NO when inflammation was induced by LPS after treatment. Interestingly, the results of the 2x and 4x dilutions of the phenolic and volatile extracts were not significantly different from one another but were all significantly different from the positive control. This indicates that when used as a preventative method, the phenolic and volatile treatments at the 2x dilution (635.7 ppm and 1.8 ppm respectively) had the same treatment effect and the phenolic and volatile treatments at the 4x dilution (317.8 ppm and 900 ppb respectively) had the same treatment effect, as opposed to only having the same treatment effect at the 2x dilution when applied as a treatment after the application of LPS. It is important to note again that the volatile extract dilution concentrations were 353x lower than the respective phenolic extract dilution concentrations and worked just as well to prevent the production of NO. P-values of all

treatments compared to the positive control are listed in Appendix B, with the significant values as follows: phenolic 635.7 ppm with a p-value of <.0001, phenolic 317.8 ppm with a p-value of <.0001, volatile 1.8 ppm with a p-value of <.0001, and volatile 900 ppb with a p-value of 0.0415.

Upon statistical comparison of the two treatment strategies, applying the treatments before inducing inflammation with LPS versus applying the treatments after inducing inflammation with LPS, it was clear that applying the treatments before inducing inflammation with LPS resulted in lower NO levels in all cases (Figure 5.3 and Figure 5.4). This indicates that the phenolic and volatile extract treatments worked better as a preventative treatment for inflammation rather than a treatment for pre-existing inflammation.

The results of the anti-inflammatory effect of the phenolic extract agreed with other studies where the total cranberry phenolic fraction or a specific portion of the total phenolic fraction tested on other cell lines, all exhibited an anti-inflammatory effect (La et al. 2010, Bodet et al. 2006, Feghali et al. 2012). There has been little to no research conducted on volatile compounds and their anti-inflammatory capabilities *in vitro,* so there is not any direct literature to compare the anti-inflammatory effect of the volatile extract.

The mechanisms of how the phenolic and volatile treatments work to prevent and treat inflammation are unclear. From the Griess Reagent System assay used in this study, only the levels of NO produced were measured, i.e. the amount of a specific inflammatory marker that is present. One possible reason for why cranberry phenolic compounds exhibit anti-inflammatory effects is their ability to inhibit cells from producing pro-inflammatory cytokines including interleukin-8 (IL-8) and chemokine ligand 5 (CCL5) and to reduce the activation of the nuclear factor-κB (NF-κB) p65 pathway when inflammation is induced by LPS (Bodet et al. 2006). Another study attributed the anti-inflammatory capabilities of cranberry extract to its' high

antioxidant capacity, showing the extract inhibited κB kinase β, which is a central node in inflammatory signal transduction (Heim et al. 2012). Similar mechanisms to those reported in the literature could have occurred in this study following treatment with the cranberry phenolic extract, however the studies used different cell lines and possibly used different types of cranberries. The volatile compounds could be reducing the aforementioned pathways and cytokines in the same way, but further research is necessary to uncover that information. Interestingly, the antioxidant activity of the volatile extract 40 μM/Kg was much lower than that of the phenolic fraction, 4676 μM/Kg, indicating that suppression of oxidative stress may not play an important role in prevention of inflammation by the volatile fraction.

The NO assay has limitations, which are important to consider. Number one, the doses of the cranberry phenolic and volatile extracts that were used to treat the RAW 264.7 cells were lower than the actual concentration in a cranberry. However, the dilutions were necessary due to needs of the RAW 264.7 cells for media and nutrients. Even at these dilutions, some of the phenolic and volatile extract treatments showed a significant anti-inflammatory effect on the RAW 264.7 cells compared to the positive control, when treated both before and after applying LPS, especially at the 2x dilution (635.7 ppm phenolic and 1.8 ppm volatile) of both extracts. Taking into consideration that cranberries can be consumed fresh or without the dilutions required for this study, it is reasonable to hypothesize an even stronger anti-inflammatory effect could be observed in an *in vivo* study.

A second limitation of this study was the quantification of only one inflammatory marker. Other biological markers of inflammation include interleukin-6, TNF-α, and C-reactive protein (Kalogeropoulos et al. 2010) It would have been beneficial to have measured the interleukin-6 levels using an IL-6 Enzyme Linked ImmunoSorbent Assay Kit, TNF-α levels using a TNF-α

Enzyme Linked ImmunoSorbent Assay Kit, and C-reactive protein levels using a CRP Quantikine Enzyme Linked ImmunoSorbent Assay kit, however, these assays were not performed due to time constraints.

Another area of limitation in this study was that the RAW 264.7 cell line used was a mouse macrophage cell line, not a human cell line, so possible differences could be observed in a human cell line. Also, this study was conducted *in vitro*, and the *in vivo* effects could be different if a similar study was conducted on live mice or humans. The current literature is deficient in the overall bioavailability of volatile compounds, and their ability to reach macrophage cells after ingestion from a food source, such as cranberries. A future study involving a lung cell line could be beneficial because volatile compounds have been previously reported to be absorbed and retained at close to the original dose in the lungs during inhalation (Kohlert et al. 2000).

Although this anti-inflammatory study had limitations, there were also strengths. Using multiple different concentrations of the phenolic and volatile extract treatments to see if the dose was relevant was beneficial to see the minimum amount of treatment required to get a significant change in the NO level. Another strength of this study included starting with the respective concentrations of phenolic and volatile compounds present in a fresh cranberry, even though they were not the same, to mimic the effects of the amount of phenolic and volatile compounds available from consuming a fresh cranberry. Testing the phenolic and volatile extracts at equivalent concentrations may have yielded lower levels of NO with the volatile extract, but due to the 353x lower concentration of volatile compounds compared to the concentration of phenolic compounds in cranberries, testing them in the manner used in this study mimics the amounts of the compounds that would be potentially available *in vivo* after consuming a cranberry.

Chapter 6: Anti-Inflammatory Effect of α-terpineol, Linalool, Linalool oxide, and Eucalyptol on RAW 264.7 cells *in vitro.*

6.1 Introduction

A secondary objective of this study was to explore the anti-inflammatory capabilities of four of the most abundant individual volatile compounds found in cranberry volatile extract, α-terpineol, linalool, linalool oxide, and eucalyptol, *in vitro* using the Griess Reagent System NO assay. The purpose of exploring each of these individual volatile compounds for their anti-inflammatory capabilities, rather than the anti-inflammatory capabilities of the entire extract, was to see if certain individual volatile compounds play a larger role in reducing inflammation than others. Treatment dosages for this objective were determined in the same manner as all other treatment dosages in this study, starting from each of the individual concentrations in the total volatile extract, 2x, 4x, and 8x dilutions were prepared. Actual concentrations of each applied treatment are shown in Table 6.1.

Table 6.1 Concentrations of individual volatile compounds applied as treatments to RAW 264.7 Cells

Treatment	[1]Actual Concentration	2x dilution	4x dilution	8x dilution
Alpha-terpineol	2320.6 ppb	1160.3 ppb	580.2 ppb	290.1 ppb
Linalool oxide	224.4 ppb	112.2 ppb	56.1 ppb	28.1 ppb
Eucalyptol	189.0 ppb	94.5 ppb	47.3 ppb	23.7 ppb
Linalool	41.3 ppb	20.7 ppb	10.3 ppb	5.2 ppb

[1]Actual concentration refers to starting concentration in total volatile extract

6.2 Materials

All standard compounds α-terpineol, linalool, linalool oxide, and eucalyptol were purchased from Sigma Aldrich (St. Louis, MO). The Griess Reagent System NO assay kit was purchased from Promega Corporation (Madison, WI).

6.3 Methods

6.3.1 Cell Culture

RAW 264.7 mouse macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified eagle's medium (DMEM) enriched with 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum (FBS). The cells were maintained in 75 cm^2 cell culture flasks and incubated at 37 °C in a 5% CO_2 environment. All NO assays were conducted between cell passage numbers 4-8. The components for the DMEM media and all experimental reagents were purchased from Gibco® through Life Technologies (Carlsbad, CA).

6.3.2 Griess Reagent System Assay (NO Assay)

Nitric oxide production was analyzed using the Griess Reagent System kit containing nitrite standard, N-1-napthylethylenediamine dihydrochloride (NED) solution, and sulfanilamide solution, which was purchased from Promega Corporation (Madison, WI). 100 µL of RAW 264.7 cells in enriched DMEM media were seeded in a 96 well plate (plate 1) and incubated at 37 °C and 5% CO_2 for 16 hr. After 16 hr, which allowed the cells to attach to bottom of the wells in the plate, the media was removed and test media containing different concentrations of α-terpineol, linalool, linalool oxide, and eucalyptol was added. The treatment media was left on the cells for 1 hr, and then removed. Next, lipopolysaccharide (LPS) media, concentration 100 ng LPS per mL, was applied to the cells and left on for 24 hr. After 24 hr, the LPS media was removed and the nitric oxide levels were measured. To measure the nitric oxide, a nitrite standard reference curve, with concentrations ranging from 0 to 100 µM, was prepared on a separate 96 well plate (plate 2). Samples on plate 1 were centrifuged for 1 min at 1000 rpm and the supernatants from the samples were added to plate 2. Then, 50 µL of sulfanilamide solution

was added to the standard reference curve and the samples on plate 2 and allowed to sit for 10 min protected from light. After ten minutes, 50 μL of NED solution was added to all wells on plate 2 and allowed to sit protected from light for 10 min. After 10 min, the absorbance was read on a plate reader at 540 nm. After the absorbance readings, corrections were made to account for background absorbance of the sample control media. The absorbance readings were converted into nitric oxide levels using the slope value from the nitric oxide standard curve.

This experiment was conducted as described above, with the α-terpineol, linalool, linalool oxide, and eucalyptol treatments applied before the LPS, and the reverse, with the treatments applied after the LPS. For the reverse, the RAW 264.7 cells were seeded in the 96 well plate and allowed to sit for 16 hr. After 16 hr the media was removed and the LPS media was applied. The LPS media was left on the cells for 24 hr. After 24 hr the α-terpineol, linalool, linalool oxide, and eucalyptol treatment medias at different concentrations were added. After 1 hr with the treatment media, the plate was centrifuged, the supernatant transferred to a second plate, and the nitric oxide levels measured in the same way as described when treatments were applied before LPS.

6.4 Statistical Analysis

All statistical analysis was performed using JMP® Pro Ver. 14 Statistics Software. One-way analysis of variance for all responses for all treatment dilution combinations before and after by each treatment combination was ran in the Fit Y by X platform of JMP Pro Ver. 14. Multiple comparisons were performed using the Tukey HSD test.

Paired t-tests were used in the Matched Pair platform of JMP Pro Ver. 14 to compare the before and after LPS results for each treatment.

6.5 Results

6.5.1 Standard Treatments after Applying LPS

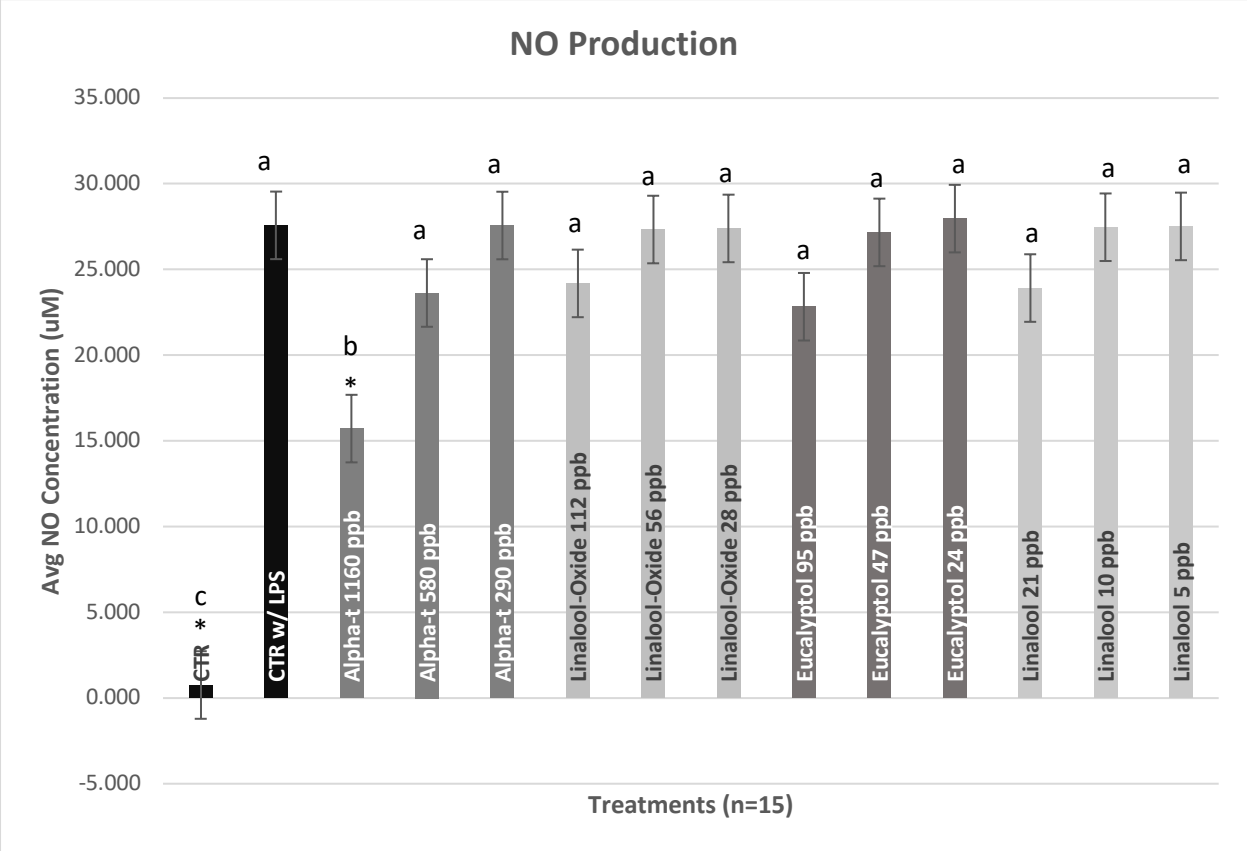


Figure 6.1 Average NO concentration produced by RAW 264.7 cells treated with α-terpineol, linalool, linalool oxide, and eucalyptol standards after applying LPS. * indicates a statistically significant difference from the positive control (CTR w/ LPS). Levels not connected by the same letter are significantly different.

Table 6.2.1 Means Comparisons of all pairs standard treatments after LPS using Tukey-Kramer HSD Confidence Quantile

	Eucalyptol -8x	Control -0x+	Alpha-Terpineol -8x	Linalool -8x	Linalool -4x	Linalool -oxide-8x	Linalool -oxide-4x
Eucalyptol -8x	-6.658	-6.265	-6.257	-6.204	-6.156	-6.085	-6.022
Control-0x+	-6.265	-6.658	-6.649	-6.597	-6.548	-6.478	-6.414
Alpha-Terpineol-8x	-6.257	-6.649	-6.658	-6.606	-6.557	-6.487	-6.423
Linalool-8x	-6.204	-6.597	-6.606	-6.658	-6.61	-6.539	-6.476
Linalool-4x	-6.156	-6.548	-6.557	-6.61	-6.658	-6.588	-6.524
Linalool-oxide-8x	-6.085	-6.478	-6.487	-6.539	-6.588	-6.658	-6.594
Linalool-oxide-4x	-6.022	-6.414	-6.423	-6.476	-6.524	-6.594	-6.658
Eucalyptol -4x	-5.853	-6.246	-6.255	-6.307	-6.356	-6.426	-6.49
Linalool-oxide-2x	-2.878	-3.271	-3.279	-3.332	-3.38	-3.451	-3.514
Linalool-2x	-2.608	-3.001	-3.009	-3.062	-3.11	-3.181	-3.244
Alpha-Terpineol-4x	-2.318	-2.711	-2.72	-2.772	-2.821	-2.891	-2.955
Eucalyptol -2x	-1.518	-1.91	-1.919	-1.972	-2.02	-2.09	-2.154
Alpha-Terpineol-2x	5.592	5.199	5.19	5.138	5.089	5.019	4.955
Control-0x-	20.552	20.159	20.151	20.098	20.05	19.979	19.916

Positive values show pairs of means that are statistically significant. Dilution factors of 2x, 4x, and 8x shown after standard name have the following concentrations respectively, alpha-terpineol 1160.3 ppb, 580.2 ppb, 290.1 ppb, linalool-oxide 112.2 ppb, 56.1 ppb, 28.1 ppb, eucalyptol 94.5 ppb, 47.3 ppb, 23.7 ppb, linalool 20.7 ppb, 10.3 ppb, 5.2 ppb.

Table 6.2.2 Means Comparisons of all pairs standard treatments after LPS using Tukey-Kramer HSD Confidence Quantile

	Eucalyptol-4x	Linalool-oxide-2x	Linalool-2x	Alpha-Terpineol-4x	Eucalyptol-2x	Alpha-Terpineol-2x	Control-0x-
Eucalyptol-8x	-5.853	-2.878	-2.608	-2.318	-1.518	5.592	20.552
Control-0x+	-6.246	-3.271	-3.001	-2.711	-1.91	5.199	20.159
Alpha-Terpineol-8x	-6.255	-3.279	-3.009	-2.72	-1.919	5.19	20.151
Linalool-8x	-6.307	-3.332	-3.062	-2.772	-1.972	5.138	20.098
Linalool-4x	-6.356	-3.38	-3.11	-2.821	-2.02	5.089	20.05
Linalool-oxide-8x	-6.426	-3.451	-3.181	-2.891	-2.09	5.019	19.979
Linalool-oxide-4x	-6.49	-3.514	-3.244	-2.955	-2.154	4.955	19.916
Eucalyptol-4x	-6.658	-3.682	-3.413	-3.123	-2.322	4.787	19.747
Linalool-oxide-2x	-3.682	-6.658	-6.388	-6.099	-5.298	1.811	16.772
Linalool-2x	-3.413	-6.388	-6.658	-6.369	-5.568	1.542	16.502
Alpha-Terpineol-4x	-3.123	-6.099	-6.369	-6.658	-5.857	1.252	16.212
Eucalyptol-2x	-2.322	-5.298	-5.568	-5.857	-6.658	0.451	15.412
Alpha-Terpineol-2x	4.787	1.811	1.542	1.252	0.451	-6.658	8.302
Control-0x-	19.747	16.772	16.502	16.212	15.412	8.302	-6.658

Positive values show pairs of means that are statistically significant. Dilution factors of 2x, 4x, and 8x shown after standard name have the following concentrations respectively, alpha-terpineol 1160.3 ppb, 580.2 ppb, 290.1 ppb, linalool-oxide 112.2 ppb, 56.1 ppb, 28.1 ppb, eucalyptol 94.5 ppb, 47.3 ppb, 23.7 ppb, linalool 20.7 ppb, 10.3 ppb, 5.2 ppb.

6.5.2 Standard Treatments before Applying LPS

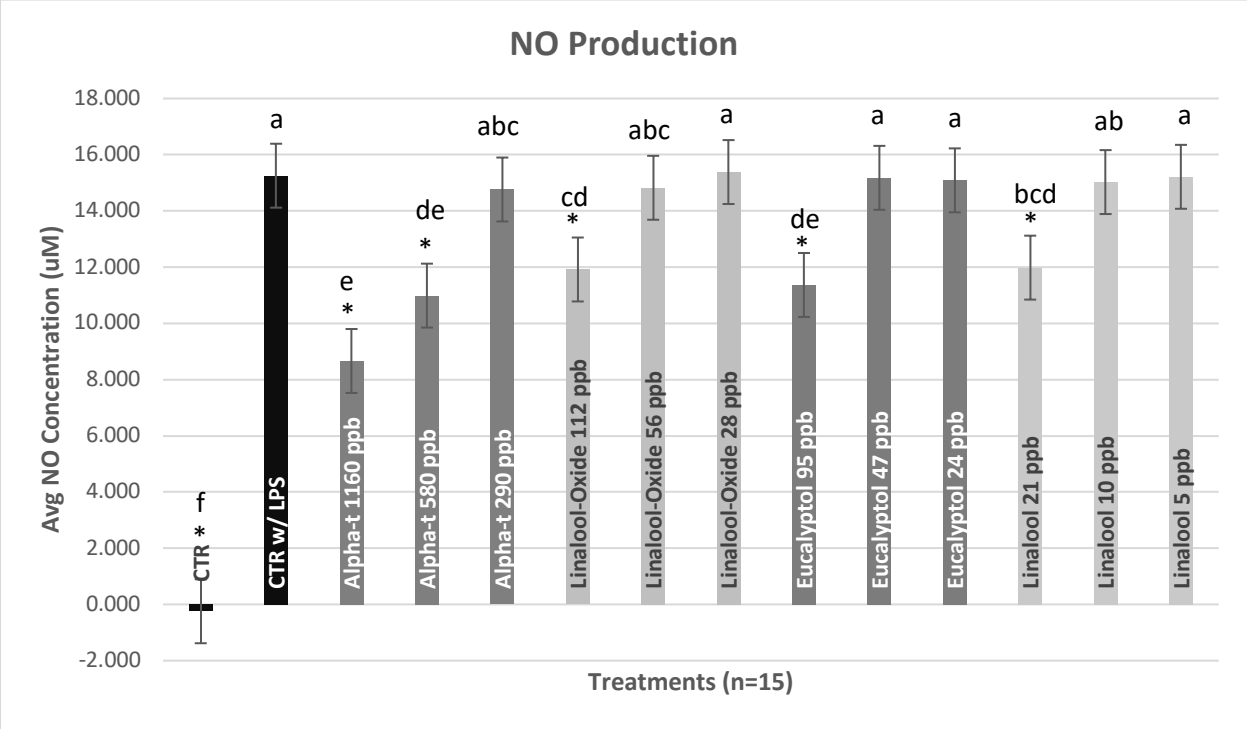


Figure 6.2 Average NO concentration produced by RAW 264.7 cells treated with α-terpineol, linalool, linalool oxide, and eucalyptol standard treatments before applying LPS. * indicates a statistically significant difference from the positive control (CTR w/ LPS). Levels not connected by the same letter are significantly different.

Table 6.3.1 Means Comparisons of all pairs of standard treatments before LPS using Tukey-Kramer HSD Confidence Quantile

	Linalool-oxide-8x	Control-0x+	Linalool-8x	Eucalyptol-4x	Eucalyptol-8x	Linalool-4x	Linalool-oxide-4x
Linalool-oxide-8x	-3.054	-2.924	-2.885	-2.848	-2.759	-2.697	-2.494
Control-0x+	-2.924	-3.054	-3.015	-2.978	-2.889	-2.827	-2.624
Linalool-8x	-2.885	-3.015	-3.054	-3.018	-2.929	-2.867	-2.664
Eucalyptol-4x	-2.848	-2.978	-3.018	-3.054	-2.965	-2.903	-2.7
Eucalyptol-8x	-2.759	-2.889	-2.929	-2.965	-3.054	-2.992	-2.789
Linalool-4x	-2.697	-2.827	-2.867	-2.903	-2.992	-3.054	-2.851
Linalool-oxide-4x	-2.494	-2.624	-2.664	-2.7	-2.789	-2.851	-3.054
Alpha-Terpineol-8x	-2.433	-2.563	-2.603	-2.639	-2.728	-2.79	-2.993
Linalool-2x	0.347	0.217	0.177	0.141	0.051	-0.011	-0.213
Linalool-oxide-2x	0.413	0.283	0.243	0.207	0.118	0.056	-0.147
Eucalyptol-2x	0.964	0.834	0.794	0.758	0.668	0.606	0.404
Alpha-Terpineol-4x	1.342	1.213	1.173	1.137	1.047	0.985	0.783
Alpha-Terpineol-2x	3.667	3.538	3.498	3.462	3.372	3.31	3.108
Control-0x-	12.573	12.443	12.403	12.367	12.278	12.216	12.013

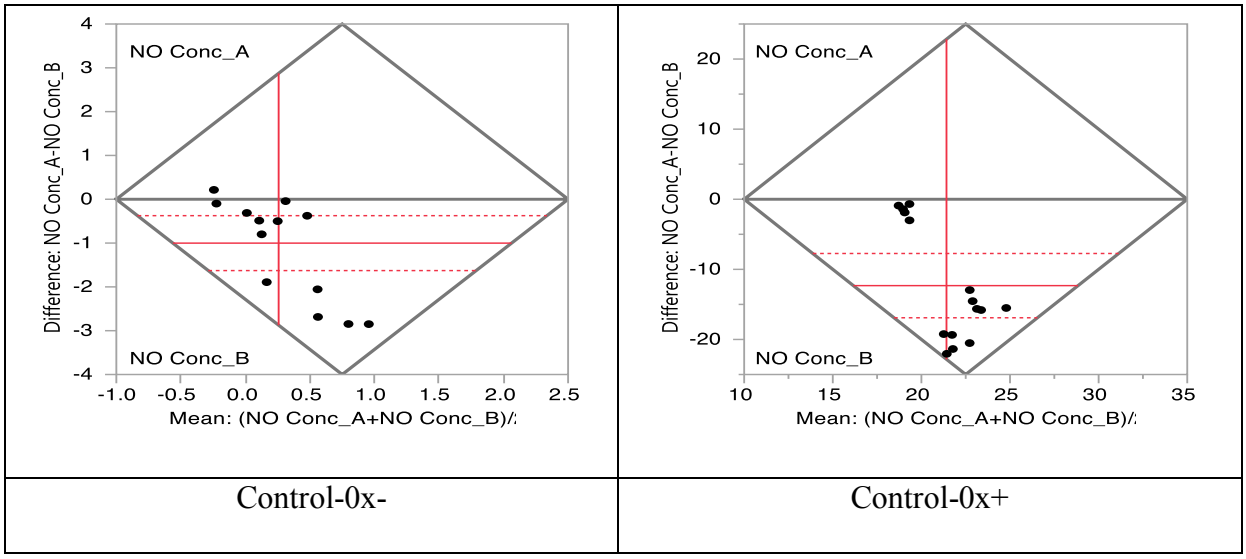
Positive values show pairs of means that are statistically significant. Dilution factors of 2x, 4x, and 8x shown after standard name have the following concentrations respectively, alpha-terpineol 1160.3 ppb, 580.2 ppb, 290.1 ppb, linalool-oxide 112.2 ppb, 56.1 ppb, 28.1 ppb, eucalyptol 94.5 ppb, 47.3 ppb, 23.7 ppb, linalool 20.7 ppb, 10.3 ppb, 5.2 ppb.

Table 6.3.2 Means Comparisons of all pairs of standard treatments before LPS using Tukey-Kramer HSD Confidence Quantile.

	Alpha-Terpineol-8x	Linalool-2x	Linalool-oxide-2x	Eucalyptol-2x	Alpha-Terpineol-4x	Alpha-Terpineol-2x	Control-0x-
Linalool-oxide-8x	-2.433	0.347	0.413	0.964	1.342	3.667	12.573
Control-0x+	-2.563	0.217	0.283	0.834	1.213	3.538	12.443
Linalool-8x	-2.603	0.177	0.243	0.794	1.173	3.498	12.403
Eucalyptol-4x	-2.639	0.141	0.207	0.758	1.137	3.462	12.367
Eucalyptol-8x	-2.728	0.051	0.118	0.668	1.047	3.372	12.278
Linalool-4x	-2.79	-0.011	0.056	0.606	0.985	3.31	12.216
Linalool-oxide-4x	-2.993	-0.213	-0.147	0.404	0.783	3.108	12.013
Alpha-Terpineol-8x	-3.054	-0.275	-0.208	0.342	0.721	3.046	11.952
Linalool-2x	-0.275	-3.054	-2.988	-2.437	-2.058	0.267	9.172
Linalool-oxide-2x	-0.208	-2.988	-3.054	-2.503	-2.125	0.2	9.106
Eucalyptol-2x	0.342	-2.437	-2.503	-3.054	-2.675	-0.35	8.555
Alpha-Terpineol-4x	0.721	-2.058	-2.125	-2.675	-3.054	-0.729	8.176
Alpha-Terpineol-2x	3.046	0.267	0.2	-0.35	-0.729	-3.054	5.851
Control-0x-	11.952	9.172	9.106	8.555	8.176	5.851	-3.054

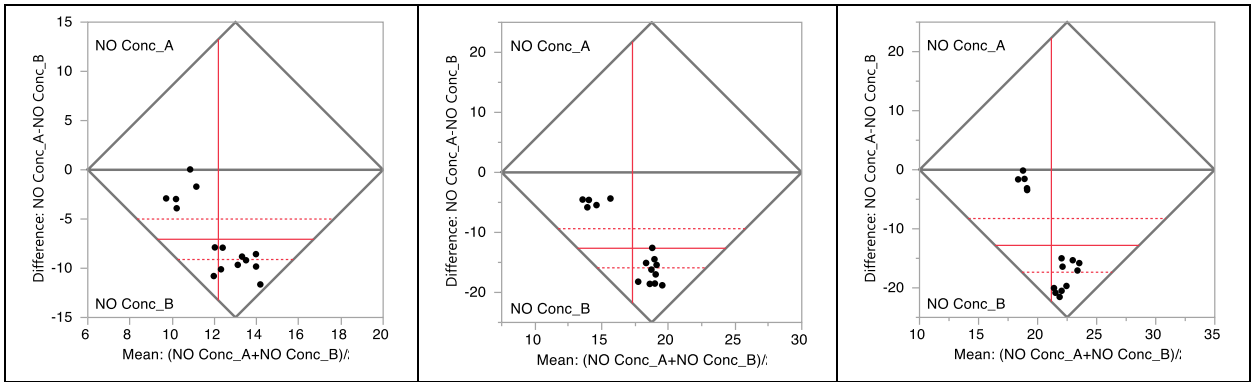
Positive values show pairs of means that are statistically significant. Dilution factors of 2x, 4x, and 8x shown after standard name have the following concentrations respectively, alpha-terpineol 1160.3 ppb, 580.2 ppb, 290.1 ppb, linalool-oxide 112.2 ppb, 56.1 ppb, 28.1 ppb, eucalyptol 94.5 ppb, 47.3 ppb, 23.7 ppb, linalool 20.7 ppb, 10.3 ppb, 5.2 ppb.

6.5.3 Analysis of Treating Before vs. Treating After Applying LPS



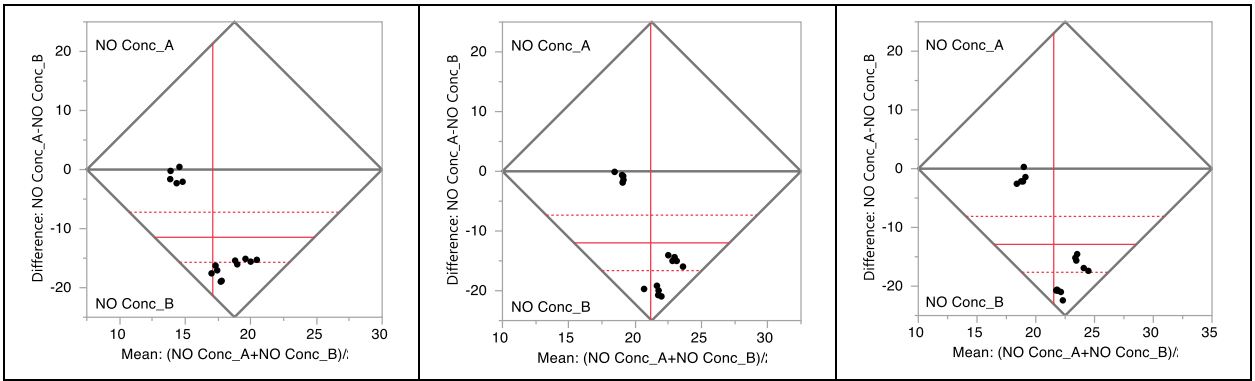
Control-0x-	Control-0x+

Figure 6.3 Comparison of NO levels of controls in experiments before and after LPS. Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.



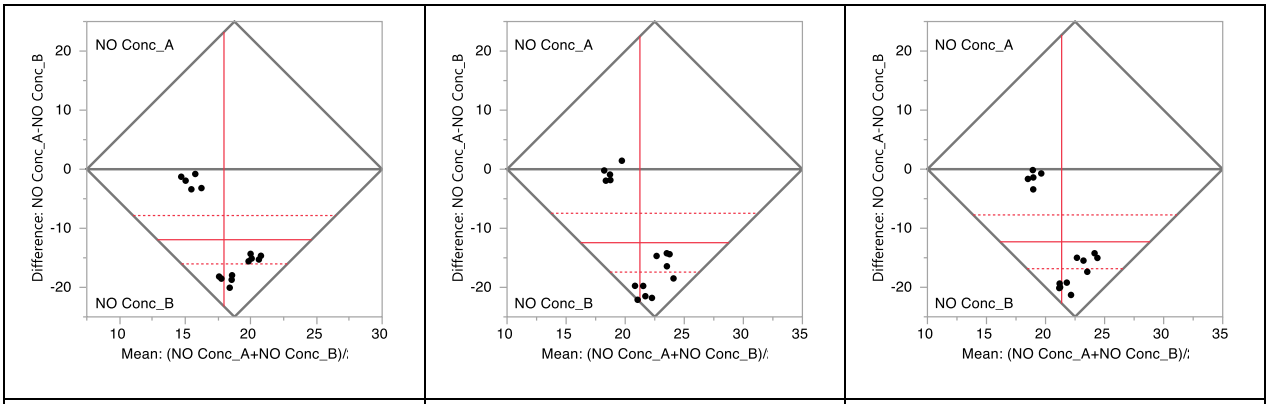
Alpha-Terpineol 1160.3 ppb	Alpha-Terpineol 580.2 ppb	Alpha-Terpineol 290.1 ppb

Figure 6.4 Comparison of NO levels treating with alpha-terpineol before and after LPS. NO_Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.



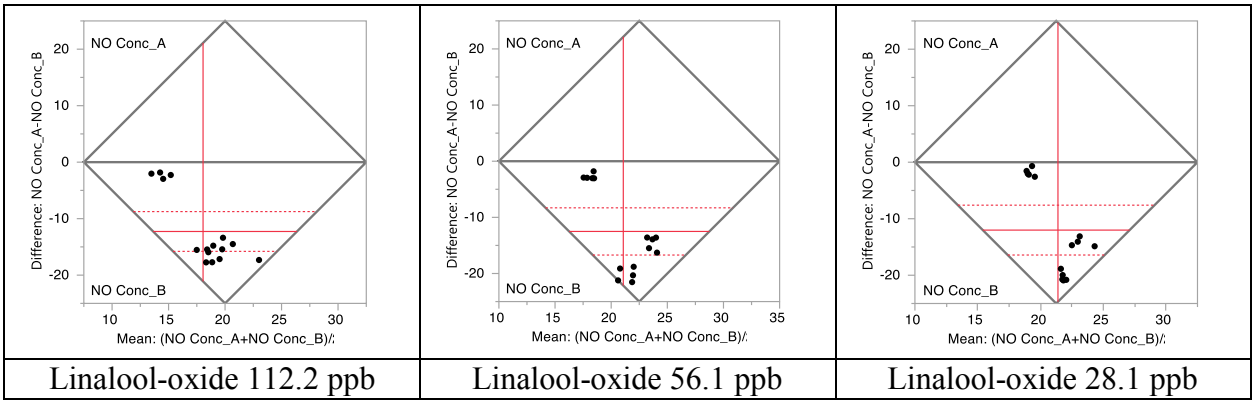
Eucalyptol 94.5 ppb	Eucalyptol 47.3 ppb	Eucalyptol 23.7 ppb

Figure 6.5 Comparison of NO levels treating with eucalyptol before and after LPS. NO_Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.



Linalool 20.7 ppb	Linalool 10.3 ppb	Linalool 5.2 ppb

Figure 6.6 Comparison of NO levels treating with linalool before and after LPS. NO_Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.



Linalool-oxide 112.2 ppb	Linalool-oxide 56.1 ppb	Linalool-oxide 28.1 ppb

Figure 6.7 Comparison of NO levels treating with linalool-oxide before and after LPS. NO_Conc_A = treatment after LPS, NO Conc_B = treatment before LPS.

6.6 Discussion

Results from the individual volatile standard treatment NO assays show potential for α-terpineol, linalool, linalool oxide, and eucalyptol to act individually as anti-inflammatory agents, especially α-terpineol. The hypothesis of this secondary objective was that each of the individual volatile standards would have an anti-inflammatory effect compared to the positive control. The results of the volatile standard treatment NO assays will be discussed in two parts, first with the volatile standard treatments applied after inflammation was induced by LPS. Figure 6.1 along with the data listed in Table 6.2.1 and Table 6.2.2 show that with application of the volatile standard treatments after applying LPS compared to the positive control, α-terpineol at 1160.3 ppb was the only treatment that exhibited a statistically significant lower amount of NO with a p-value of <.0001, p-values of all treatments compared to the positive control are listed in Appendix B. These results are likely due to the dilutions of α-terpineol from the starting concentration being the highest of all the standard compounds because it was found to have the highest individual concentration in the cranberry total volatile extract.

Second, the results of the volatile standard treatments of the cells before applying LPS supported the hypothesis that each of the individual volatile compounds would have an anti-inflammatory effect compared to the positive control. Figure 6.2 and with the data listed in Table 6.3.1 and Table 6.3.2 show that with application of volatile standard treatments before applying LPS compared to the positive control, i.e. looking at a preventative effect, NO levels decreased after treatment by α-terpineol at 1160.3 ppb, α-terpineol at 580.2 ppb, linalool at 20.7 ppb, linalool-oxide at 112.2 ppb, and eucalyptol at 94.5 ppb. A relevant dose response is also shown in the results when the experiment was conducted in this manner. As the dilution factor of the volatile standard treatments increased, the significance of the NO levels compared to the positive

control was reduced, showing that the more concentrated treatments were more effective in preventing the cells from producing as much NO when inflammation was induced by LPS after treatment. The α-terpineol 1160.3 ppb exhibited significantly lower NO levels that all of the other treatments that were statistically significant from the positive control, and the α-terpineol 580.2 ppb was not significantly different from linalool at 20.7 ppb, linalool-oxide at 112.2 ppb, or eucalyptol at 94.5 ppb, indicating that all of those concentrations of volatile standard treatments had the same preventative anti-inflammatory capabilities. P-values of all treatments compared to the positive control are listed in Appendix B, with the significant values as follows: α-terpineol 1160.3 ppb with a p-value of <.0001, α-terpineol 580.2 ppb with a p-value of 0.0003, eucalyptol 94.5 ppb with a p-value of 0.0019, linalool-oxide 112.2 ppb with a p-value of 0.0184, and linalool 20.7 ppb with a p-value of 0.0235.

Upon statistical comparison of the two treatment strategies, applying the treatments before inducing inflammation with LPS versus applying the treatments after inducing inflammation with LPS, applying the treatments before inducing inflammation with LPS resulted in lower NO levels in all cases (Figures 6.3 - 6.7). This indicated that the volatile standard treatments worked better as a preventative treatment for inflammation rather than a treatment for pre-existing inflammation.

The standard α-terpineol exhibited the most effective anti-inflammatory effects on the RAW 264.7 cells in this anti-inflammatory assay, being the only individual standard to work as treatment to pre-existing NO and as a treatment to prevent NO production and lowering NO levels compared to the control by 43.0% when applied after LPS and 43.3% when applied before LPS. There is no other current data reporting the anti-inflammatory effects of α-terpineol on RAW 264.7 cells for comparison, however α-terpineol has been shown to have anti-

inflammatory effects on other cell lines. In a study looking at epithelial buccal cells, α-terpineol from orange juice was found to have an anti-inflammatory effect by reducing IL-6 production by inhibiting the gene expression of the IL-6 receptor (Held et al. 2007). In another study on U937 human macrophage cells looking at tea tree oil steam distilled from *Melaleuca alternifolia* that contains α-terpineol, the inflammation markers IL-β1, IL-6, and IL-10 induced by LPS were found to be significantly reduced by treatment of the cells with the tea tree oil, and the mechanism for the inhibition of the inflammatory markers was that the tea tree oil extract components, specifically noted as α-terpineol and terpinen-4-ol, interfered with the NF-κB, p38, extracellular signal-regulated kinase (ERK), or mitogen-activated protein kinase (MAPK) pathways (Nogueira et al. 2014). In the present study, it is possible that these same pathways were inhibited upon treatment with α-terpineol, but the differences in cell lines and source of the α-terpineol must be considered upon comparison.

The standard eucalyptol exhibited an anti-inflammatory effect in the present study only when applied to the RAW 264.7 cells as a treatment before inducing inflammation with LPS, lowering NO levels by 25.4% compared to the positive control. There is no other current data reporting the anti-inflammatory effects of eucalyptol on RAW 264.7 cells for comparison, however eucalyptol has been shown to have anti-inflammatory effects. In an *in vivo* study on mice, eucalyptol was found to inhibit the production of TNF-α, IL-β1, and IL-6 induced by injection of complete Freund's adjuvants, CFA, by a mechanism dealing with the transient receptor potential cation channel subfamily M member 8, TRPM8, (Caceres et al. 2017). In another study by Caceres Bustos et al. (2016), working on lung cells of mice, LPS was used to induce inflammation, and after 24 hr, the lungs and bronchoalveolar lavage of eucalyptol treated and untreated TRPM8 knockout and TRPM8 wild-type were taken to be analyzed and found that

only the TRPM8 wild-type mice exhibited anti-inflammatory effects, showing that TRPM8 was essential for eucalyptol to exhibit its anti-inflammatory capabilities. In another study involving eucalyptol and inflammation on a different cell type, but similar time frame and induction of inflammation to the present study, mice were intraperitoneally injected with eucalyptol one hr before being challenged with LPS and compared to controls. The eucalyptol injected mice had lower levels of TNF-α, IL-6, NO, and NF-κB (Kim et al. 2015). These studies support the data in the present study that eucalyptol works as a suppressor of anti-inflammatory markers when used as a pre-treatment to induced inflammation, and also that the mechanism for the anti-inflammatory properties of eucalyptol in this study could have involved TRPM8, however comparisons between the studies must be done with caution due to the use of different cell lines. Confirmation of the mechanisms and suppression of other anti-inflammatory markers besides NO on RAW 264.7 cells warrants future research.

The standard linalool-oxide exhibited an anti-inflammatory effect in the present study only when applied to the RAW 264.7 cells as a treatment before inducing inflammation with LPS, lowering NO levels by 21.8% compared to the positive control. There is no other current data reporting the anti-inflammatory effects of linalool-oxide for comparison, therefore the mechanisms and the ability to suppress other anti-inflammatory markers in RAW 264.7 cells warrants future research.

The standard linalool exhibited an anti-inflammatory effect in the present study only when applied to the RAW 264.7 cells as a treatment before inducing inflammation with LPS, lowering NO levels by 21.4% compared to the positive control. There is one other current study reporting the anti-inflammatory effects of linalool on RAW 264.7 cells for comparison. Huo et al. (2013) investigated the preventative effect of linalool *in vitro* on RAW 264.7 cells and *in vivo*

on a lung injury model and found that linalool lowered the production of TNF-α, and IL-6 *in vitro* and *in vivo* compared to controls. This study looked at different biological markers of inflammation than the present study but confirms the preventative effect of linalool on RAW 264.7 cells as a treatment for inflammation. There are other studies that have looked at linalool and the anti-inflammatory effect. In a study evaluating the anti-inflammatory effects of linalool from *Cinnamomum osmophloeum Kanehira*, a Taiwan native plant, mice were administered linalool at 2.6 and 5.2 mg per kg of body weight before injected with endotoxin to induce inflammation, and the mice treated with linalool were found to have decreased levels of the inflammatory markers peripheral nitrate and nitrite, IL-1β, IL-18, TNF-α, and IFN-λ (Lee et al. 2018) Contrary to the anti-inflammatory effect found in the present study and other current studies, in the study by Held et al. (2007) linalool identified in orange juice was found to have no effect on the production of the pro-inflammatory cytokine IL-6, and was found to have no inhibition of gene expression of the IL-6 receptor in epithelial buccal cells and overall no anti-inflammatory effect. However, the results from this study were found using linalool isolated from orange juice and evaluated on a different cell line, which could have led to the differing results. The mechanisms by which linalool acts as an anti-inflammatory treatment on RAW 264.7 cells has yet to be determined and warrants further research.

The NO assay used for experimentation in the present study had some limitations. First, it is hard to compare the effects of the treatments of α-terpineol, linalool, linalool oxide, and eucalyptol directly with each other because they were applied as treatments on the RAW 264.7 cells at different concentrations. However, different concentrations were necessary to keep consistent with the rest of the study and to be as representative as possible of the concentrations of these compounds found in a fresh cranberry. Second, it is hard to determine if the results of

this study would translate *in vivo* in humans, because a mouse cell line was used and the ability of α-terpineol, linalool, linalool oxide, and eucalyptol to get into macrophages in humans has not been determined and warrants future research. Third, the exact mechanisms by which α-terpineol, linalool, linalool oxide, and eucalyptol provided their respective anti-inflammatory effects in this study were not tested, and other experimental assays along with the NO assay could have been conducted to determine levels of other inflammatory markers.

Chapter 7: Final Conclusions

Three objectives were addressed in the present study, number one was to extract, quantify and identify the volatile and phenolic compounds in cranberries. Number two was to compare the anti-inflammatory effect of the volatile and phenolic extracts from cranberries on RAW 264.7 mouse macrophage cells, and number three was to compare the anti-inflammatory effect of four individual, more abundant volatile compounds from cranberries, α-terpineol, eucalyptol, linalool oxide, and linalool, on RAW 264.7 mouse macrophage cells.

For objective number one, the Folin-Ciocalteu assay and HPLC were used. The total phenolic content of the cranberries in this study was 1271 mg GAE equiv. per kg fresh weight. Using HPLC, the concentration of total anthocyanins was found to be 936.6 ± 8.0 mg ACY-3-glu equiv. per kg of fresh weight, the concentration of total flavonols was found to be 84.4 ± 1.0 mg rutin equiv. per kg of fresh weight, and the concentration of total hydroxycinnamic acids was found to be 14.7 ± 0.0 mg chlorogenic acid equiv. per kg of fresh weight. The total volatile content of the cranberries used in this study was 3551.5 ppb. Using GC-MS, 25 individual volatile compounds were identified, with the more prevalent individual compounds being α-terpineol at 2321 ppb, linalool oxide at 224 ppb, eucalyptol at 189 ppb, and linalool at 41 ppb,

which agreed with other cranberry studies finding α-terpineol to make up the majority of the volatile fraction of cranberries.

For objective number two, the Griess Reagent System assay was used to measure NO levels produced *in vitro* by RAW 264.7 cells when treated with volatile and phenolic extracts before and after inducing inflammation using LPS. When the cells were treated with phenolic and volatile extracts after inducing inflammation with LPS, the phenolic extract treatments and the 1.8 ppm volatile extract treatment showed statistically significant, lower NO levels compared to the positive control. When the cells were treated with phenolic and volatile extracts before inducing inflammation with LPS, the 635.7 ppm and 317.8 ppm phenolic extract treatments and 1.8 ppm and 0.9 ppm volatile extract treatments showed statistically significant, lower NO levels compared to the positive control. Treating the cells with the phenolic and volatile extracts before inducing inflammation with LPS resulted in lower levels of NO in all treatments. Results indicate that the total phenolic and volatile extracts from a cranberry act as an effective preventer of inflammation and an effective treatment for pre-existing inflammation.

For the last objective, the Griess Reagent System assay was used to measure NO levels produced *in vitro* by RAW 264.7 cells when treated with α-terpineol, linalool, linalool oxide, and eucalyptol before and after inducing inflammation using LPS. When the cells were treated with α-terpineol, linalool, linalool oxide, and eucalyptol after inducing inflammation with LPS, only the 1160.3 ppb α-terpineol resulted in statistically significant, lower NO levels compared to the positive control. However, when the cells were treated before LPS, α-terpineol 1160.3 ppb and 580.2 ppb, linalool 20.7 ppb, linalool oxide112.2 ppb, and eucalyptol 94.5 ppb all showed statistically significant lower NO levels than the positive control.

Future research is warranted to determine the mechanisms by which all treatments in this study exhibited their respective anti-inflammatory effects. Uncovering the mechanisms by which these treatments lower inflammation could aid in the treatment of diseases involving inflammation such as obesity and periodontal disease. Future research is also necessary to determine the bioavailability of cranberry volatile compounds, because as of now it is unknown whether they can reach macrophage cells in humans at high enough doses to elicit the effects seen in this *in vitro* study. Other recommendations for future research would be to re-run the current study and measure additional inflammatory markers such as IL-6, TNF-α, and C-reactive protein, and to investigate the possible synergistic effects of combinations of the volatile standards, volatile extract, and phenolic extract.

In final summary, the most relevant finding of this study is that the volatile compounds extracted from cranberries have a similar anti-inflammatory effect to the phenolic compounds at a 353x lower concentration, whereas all previous studies have attributed the health benefits of cranberries to phenolic compounds. These results provide exciting information for future prevention and treatment of inflammation, as cranberries are low cost and easy to obtain in the United States. Taking into consideration that all fruits and vegetables contain some level of these volatile compounds, there are vast possibilities for future research on plant-based treatments for inflammation.

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Appendix A: Biosafety Research Approval Letter

MEMORANDUM TO:
FROM:
RE:

PROTOCOL #:

PROTOCOL TITLE: cells

APPROVED PROJECT PERIOD:

September 15, 2017

Dr. Sun-Ok Lee
Ines Pinto, Biosafety Committee Chair
New Protocol
18015
Testing the effects of extracts and bioactive components on lung cancer

Start Date September 14, 2017 Expiration Date September 13, 2020

The Institutional Biosafety Committee (IBC) has approved Protocol 18015, "Testing the effects of extracts and bioactive components on lung cancer cells". You may begin your study.

If modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

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.

CONTACT INFORMATION

Principal Investigator:

Name: Sun-Ok Lee | Title: Associate Professor

Department: FDSC | Campus Address: 2650 N. Young Ave.

Phone#: A/C [ ] Phone 5-6941 | E-Mail sunok@uark.edu

Fax#: A/C [ ] Phone 479-575-6936

After hours phone number (required if research is at Biosafety Level 2 or higher):

Phone#: A/C [ ] Phone 479-200-5610

Co-Principal Investigator:

Name: | Title:

Department: | Campus Address:

Phone#: A/C [ ] Phone | E-Mail

Fax#: A/C [ ] Phone

After hours phone number (required if research is at Biosafety Level 2 or higher):

Phone#: A/C [ ] Phone

PROJECT INFORMATION

Have you registered ANY project previously with the Institutional Bio Safety Committee? (*Check one*) [✓] Yes [ ] No

Are you registering a new project or renewing a previous project registration?

[✓] New project [ ] Renewal (*Mandatory after 3 years*)

Project Title: Testing the effects of extracts and bioactive components on lung cancer cells

Project Duration: Start Date 9/15/2017 End Date 9/14/2020

Indicate what containment conditions you propose to use (check all that apply):

Biosafety Level 1 *(2,3)*	Biosafety Level 2 *(2,3)* ✓	Biosafety Level 3 *(2,3)*
Animal Biosafety Level 1 *(2,3a,3b)*	Animal Biosafety Level 2 *(2,3a,3b)*	Animal Biosafety Level 3 *(2,3a,3b)*
Plant Biosafety Level 1 *(3)*	Plant Biosafety Level 2 *(3)*	Plant Biosafety Level 3 *(3)*

NOTE: Hyperlinks for references throughout this form are available on the last page of this form.

References for Biosafety criteria:

(1) University of Arkansas Biological Safety Manual - http://ehs.uark.edu/DocumentPages/BiosafetyManual04.pdf

(2) Biosafety in Microbiological and Biomedical Laboratories (BMBL) - 4th edition. CDC - Dept. of Health & Human Services - http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

(3) NIH Guidelines for work involving recombinant DNA molecules; "http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html"

If working at BL-2, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?

[✓] Yes Date (if known) [ ] [ ] No (*If No - schedule the inspection with the BSO*)

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	17 years of experience in conducting research involving human cell and animal tissue cultures
Cindi Brownmiller - Research Associate	20 months of experience in cell cultures
Katherine Moore-M.S. student	No experience. Get a training from Dr. Lee
Inah Gu- M.S. student	12 months of experience in cell cultures
Wing Shun Lam- M.S. student	8 months of experience in cell cultures
Brittany Frederick- M.S. student	6 months of experience in cell cultures

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.

Appendix B: P-values for all statistical analysis

Table 1 p-values of phenolic and volatile treatments after LPS compared to positive control. * indicates significance.

Level	- Level	Difference	Std Err Dif	p-Value
Control-0x+	Control-0x-	14.95807	2.141153	<.0001*
Control-0x+	Phenolic-2x	8.9912	2.141153	0.0014*
Control-0x+	Volatile-2x	7.36513	2.141153	0.0180*
Control-0x+	Phenolic-4x	6.6736	2.141153	0.0463*
Control-0x+	Volatile-4x	4.89847	2.141153	0.3096
Control-0x+	Phenolic-8x	3.39933	2.141153	0.7567
Control-0x+	Volatile-8x	3.2536	2.141153	0.7954

Table 2 p-values of phenolic and volatile treatments before LPS compared to positive control. * indicates significance.

Level	- Level	Difference	Std Err Dif	p-Value
Control-0x+	Control-0x-	22.1686	0.9497967	<.0001*
Control-0x+	Phenolic-2x	12.03447	0.9497967	<.0001*
Control-0x+	Volatile-2x	10.792	0.9497967	<.0001*
Control-0x+	Phenolic-4x	5.91233	0.9497967	<.0001*
Control-0x+	Volatile-4x	2.99787	0.9497967	0.0415*
Control-0x+	Phenolic-8x	2.47493	0.9497967	0.1649
Control-0x+	Volatile-8x	0.3998	0.9497967	0.9999

Table 3 p-values of standards treatments after LPS compared to positive control.
* indicates significance.

Level	- Level	Difference	Std Err Dif	p-Value
Control-0x+	Control-0x-	26.81753	1.960337	<.0001*
Control-0x+	Alpha-Terpineol-2x	11.8572	1.960337	<.0001*
Control-0x+	Eucalyptol-2x	4.74773	1.960337	0.4684
Control-0x+	Alpha-Terpineol-4x	3.947	1.960337	0.7575
Control-0x+	Linalool-2x	3.6574	1.960337	0.8422
Control-0x+	Linalool-oxide-2x	3.3876	1.960337	0.9035
Control-0x+	Eucalyptol-4x	0.41193	1.960337	1
Control-0x+	Eucalyptol-8x	0.39287	1.960337	1
Control-0x+	Linalool-oxide-4x	0.24367	1.960337	1
Control-0x+	Linalool-oxide-8x	0.18	1.960337	1
Control-0x+	Linalool-4x	0.10967	1.960337	1
Control-0x+	Linalool-8x	0.06113	1.960337	1
Control-0x+	Alpha-Terpineol-8x	0.00873	1.960337	1

Table 4 p-values of standards treatments before LPS compared to positive control.
* indicates significance.

Level	- Level	Difference	Std Err Dif	p-Value
Control-0x+	Control-0x-	15.49727	0.8992653	<.0001*
Control-0x+	Alpha-Terpineol-2x	6.59193	0.8992653	<.0001*
Control-0x+	Alpha-Terpineol-4x	4.26693	0.8992653	0.0003*
Control-0x+	Eucalyptol-2x	3.888	0.8992653	0.0019*
Control-0x+	Linalool-oxide-2x	3.3372	0.8992653	0.0184*
Control-0x+	Linalool-2x	3.27107	0.8992653	0.0235*
Control-0x+	Alpha-Terpineol-8x	0.49133	0.8992653	1
Control-0x+	Linalool-oxide-4x	0.43013	0.8992653	1
Control-0x+	Linalool-4x	0.22733	0.8992653	1
Control-0x+	Eucalyptol-8x	0.16533	0.8992653	1
Control-0x+	Linalool-oxide-8x	0.1298	0.8992653	1
Control-0x+	Eucalyptol-4x	0.07613	0.8992653	1
Control-0x+	Linalool-8x	0.03973	0.8992653	1