

CRANBERRY POLYPHENOLS DOWN-REGULATE THE TOLL-LIKE RECEPTOR 4
PATHWAY AND NUCLEAR FACTOR-KAPPA B ACTIVATION, WHILE STILL
ENHANCING TUMOR NECROSIS FACTOR ALPHA SECRETION IN HL-60 CELLS

By

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To my parents, Bruce and Marie Muller, and my grandparents, John and Madeline Tuite

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
µM	Micromolar
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATRA	All-trans retinoic acid
CD	Cluster designation
CT	Catechin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP-B	B-type dimeric procyanidin oligomers from cocoa
DPBS	Dulbecco's phosphate buffered saline
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC	Epicatechin
EGCG	Epigallocatechin
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related protein kinase
GI	Gastrointestinal
IκB	Inhibitor of kappa B
IκBα	Inhibitor of kappa B alpha
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IKK	Inhibitor of kappa B kinase
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's media
IRAK4	Interleukin-1 receptor-associated kinase 4
IT	Infected, treated
IU	Infected, untreated
JNK	C-jun N-terminal
LBP	Lipopolysaccharide binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
mL	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor-kappa B
ng	Nanogram
NK	Natural killer
nm	Nanometer
nM	Nanomolar
NT	Non-infected, treated
NU	Non-infected, untreated
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
pg	Picogram

plkB α	Phosphorylated inhibitor of kappa B
PMA	Phorbol myristic acid
RANTES	Regulated upon activation normal T-cell expressed and secreted
T-cell	Thymocyte
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF α	Tumor necrosis factor alpha

Abstract of Thesis Presented to the Graduate School
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Plant polyphenols have been studied extensively for their effects on immune responses. Tea and grape polyphenols have received a great deal of attention in this area of study, while the cranberry, known for its uncommon proanthocyanidin linkages and high antioxidant activity, has been given little of this attention. In this study, six cranberry polyphenol fractions were examined for antioxidant activity, their ability to increase total inhibitor of kappa B alpha (I κ B α), decrease phosphorylated I κ B α (pI κ B α) and decrease interleukin-1 receptor-associated kinase (IRAK) 4, a signaling protein in the toll-like receptor (TLR) pathway, protein expression and to decrease tumor necrosis factor alpha (TNF α) secretion in a lipopolysaccharide (LPS)-stimulated neutrophil model.

The six fractions included proanthocyanidins, anthocyanins, and other polyphenols from the presscake, as well as the presscake concentrate and proanthocyanidins and a mixture of polyphenols from the juice. HL-60 cells were used to study differentiation and were also differentiated with all-*trans* retinoic acid for use as a neutrophil model. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity

(ORAC) assays were used to measure antioxidant activity. Differentiation was assessed via the reduction of cytochrome C by superoxide anion production in response to phorbol myristate acetate. Protein levels of total and pI κ B α and IRAK4 were determined using the Western blot technique and an enzyme-linked immunosorbent assay (ELISA) was run to measure TNF α secretion.

The antioxidant activity assays showed that the proanthocyanidin-rich presscake and juice fractions and the mixed-polyphenol-enriched juice fraction had the highest activity. These three fractions were used in the protein experiments. None of the fractions caused differentiation in the HL-60 cells at 75 μ g/mL. In the neutrophil-like cells treated with LPS, the juice and presscake proanthocyanidin-rich fractions significantly reduced the amount of pI κ B α protein present, while the presscake proanthocyanidin-treated cells showed reduced IRAK4 levels compared to untreated cells stimulated with LPS. The proanthocyanidin-rich fractions from the presscake and the juice also significantly increased total I κ B α protein levels compared to untreated, LPS-stimulated cells. Interestingly, TNF α secretion was approximately 10 times higher in fraction-treated cells compared to the LPS control cells.

These results suggest that the proanthocyanidin-rich cranberry fractions can prevent the extensive inflammatory response mediated by the release of nuclear factor-kappa B (NF- κ B) from I κ B. One way these polyphenols accomplish this is likely through interfering with the TLR4 pathway as evidenced by the reduced IRAK4 expression. However, the increase in TNF α suggests that the polyphenol-rich fractions studied may also up-regulate additional, possibly more targeted, inflammatory pathways to aid in coping with the pathogenic onslaught.

CHAPTER 1 BACKGROUND

Introduction

Plants and their chemical constituents have enjoyed a great deal of attention for the health benefits they may impart. The cranberry, *Vaccinium macrocarpon*, is no exception and has a rich history in research and in folk medicine. However, cranberry's impact on immunity has been largely overlooked in the laboratory.

Due to the expanse of research on plant polyphenols, and the scarcity of work on cranberry's impact on immunity, a project to shed more light on this area was carried out. Based on the literature, it was hypothesized that polyphenol-rich cranberry fractions, which included proanthocyanidins, would be effective in encouraging differentiation of immature immune cells and that they would inhibit the toll-like receptor (TLR) 4 and nuclear factor- κ B (NF- κ B) inflammatory pathways and reduce inflammatory cytokine secretion in response to lipopolysaccharide (LPS) activation.

The Immune System

The immune system is the guardian of health. It is comprised of various cells throughout the body that interact via chemical messengers, like cytokines and chemokines. Generally, these messengers affect immune cells by binding to receptors on the cell surface and inducing a signaling cascade which results in some effect on the cell, such as production of its own cytokines. Specific immune cells and receptors are discussed in the sections below.

Cells of the Immune System

The immune system is composed of several different types of cells that function in innate or acquired immunity. The cells of the innate immune system include

neutrophils, macrophages, basophils, mast cells, eosinophils, and natural killer (NK) cells. Innate immunity is a quick acting defense against a broad range of invaders, while acquired immunity offers a slower, more specific response. Innate immune cells can migrate to the site of infection—this action is called chemotaxis—where they may use phagocytosis and respiratory burst to kill pathogens. Cytokines secreted by other immune cells prompt chemotaxis to the site of infection. This immune response may cause local inflammation, but this is generally mild and brief. However, if there is chronic inflammation due to continual immune activation, it can negatively impact health. Heart disease is one of many diseases that is, at least in part, caused by inflammation. A review by Libby and Theroux [1] of the role of inflammation in heart disease can be consulted for more detailed information.

Toll-Like Receptors

To begin the immune response, a component of the bacterium or other foreign body must be recognized as non-self. Recognition of pathogens can occur through cellular receptors, like TLRs. TLRs are cell-surface receptors that ultimately cause NF- κ B activation. Their discovery in humans was published in 1997 by Medzhitov et al. [2]. They were named for their homology to the Toll receptors first found in *Drosophila*. In *Drosophila*, Toll mediates embryo development and immunity. In mammals, TLRs are thought to be related only to immunity. There are many proteins that aid TLR in transducing its signal once an antigen has been presented to the receptor and a few of the key signaling proteins are shown in Figure 1-1. A detailed explanation of the pathway can be found in the review written by O'Neill [3]. Ultimately, the pathway leads to the inhibitor of kappa B alpha (I κ B α) being phosphorylated, which causes the dissociation of I κ B α and NF- κ B. Then, NF- κ B can move into the nucleus where it acts

as a transcription factor for numerous genes. Some of the types of genes it encourages the transcription of include cytokines, acute phase proteins, cellular adhesion molecules, stress response proteins, growth factors, other transcription factors, enzymes, and cell-surface receptors [4]

Plant Polyphenols

Polyphenols are a diverse group of compounds commonly found in plants. They include phenolics acids, benzoquinones, acetophenones, phenylpropenes, stilbenes, lignins, and flavonoids among others [5]. Flavonoids have a three-ringed structure pictured in Figure 1-2. Most commonly, they have a glucose, galactose, rhamnose, xylose, or arabinose moiety attached to their main structure [6]. Flavonoids can also be broken down into classes based on their structure and are widespread in plants [5]. Two classes of flavonoids include anthocyanins and proanthocyanidins.

Anthocyanins and Proanthocyanidins

Anthocyanins are responsible for red, blue, and purple pigments in plants [6]. Proanthocyanidins are oligomers and polymers of flavan-3-ols and generally have high molecular weights [5,7]. Catechin (CT) and epicatechin (EC) are the most common monomers of proanthocyanidins [7]. The monomers are linked either with one bond, the B-type linkage, or with two bonds, the A-type linkage, illustrated in Figure 1-3.

A-type linkages are relatively uncommon and have been characterized only in cranberries, avocado, plum, curry, cinnamon, and peanuts so far [8]. A-type linkages have been shown to have anti-viral effects against the human immunodeficiency virus and herpes simplex virus [9] and may be useful in preventing the bacterial adhesion of urinary tract infections [10,11]. The B-type linkages of proanthocyanidins are ubiquitous in nature.

Anti-inflammatory Characteristics of Polyphenols

A great deal of research has been devoted to the study of polyphenols from various plants. There is a large body of evidence for their efficacy to modulate the inflammatory process both *in vitro* and *in vivo* animal studies. This section includes a sampling of the literature that specifically involves plant polyphenols and their effects on inflammation.

Several groups have looked at polyphenols and their anti-inflammatory nature in *in vitro* situations [12-15]. One set of experiments focused on 23 polyphenol compounds from various sources, which included several anthocyanin extracts [12]. Researchers found that the polyphenols reduced nitric oxide production after RAW 264.7 macrophages were stimulated with LPS and interferon-gamma (IFN- γ). Tumor necrosis factor alpha (TNF α) levels were increased by some polyphenols and decreased by others. All anthocyanins tested increased TNF α levels. A second study looked at KU812 cells, a cancerous cell line differentiated to mast cells, and pomegranate polyphenols [13]. Cellular mRNA and interleukin (IL)-6 and IL-8 levels decreased after the cells were incubated with phorbol myristic acid (PMA) and then treated with pomegranate extract high in polyphenols, especially anthocyanins. The pomegranate extract also inhibited the phosphorylation of c-jun N-terminal (JNK) and extracellular signal-related protein kinase (ERK), the degradation of I κ B α , and the movement of NF- κ B into the nucleus. Interestingly, another group [14] showed that MOLT-4 thymocytes (T-cell) precursor cells treated with quercetin and ellagic acid activated the mitogen-activated protein (MAP) kinases JNK1, JNK2, and p38. Mackenzie et al. studied EC, CT, and B-type dimeric procyanidin (DP-B) and their effect on T-cell NF- κ B activation in response to PMA stimulation [15]. It was determined that all three test

flavonoids were transported into the cell and that EC and DP-B were also found in the nucleus of Jurkat T-cells. The three test compounds significantly inhibited the binding of NF- κ B to deoxyribonucleic acid (DNA) and the secretion of IL-2 compared to the PMA control. I κ B α phosphorylation was decreased in all three flavonoids compared to the PMA control and the inhibitor of kappa B kinase beta (IKK β) was decreased in EC and DP-B samples. There was a trend toward decreasing IKK β phosphorylation in CT, but it was not significant due to a high standard error. Cellular oxidants decreased compared to the PMA control in cells incubated with EC, CT, and DP-B. The authors also showed that the flavonoids could selectively inhibit the binding of NF- κ B to DNA without interfering with the binding of other transcription factors, like octamer transcription factor-1 or cyclic adenosine monophosphate response element binding protein.

Polyphenol-rich fractions of several cereals were examined for their effect on immune response markers in a study by Álvarez et al. [16]. Female ICR mice were fed a control diet of standard rodent diet or a diet of 80% standard diet and 20% wheat germ, buckwheat flour, fine rice bran, or wheat middlings fractions ad libitum for 5 weeks. The cereal fractions were mixed with water and formed into biscuits. Control mice were also fed biscuits made from standard diet which were prepared in a similar fashion as the cereal biscuits. In mice fed the cereal enriched diet, lymphocyte chemotaxis and proliferation in response to Concanavalin A were increased beyond that of the normal chow control mice. Lymphocyte proliferation in response to LPS stimulation was higher in all cereal enriched diets except the wheat germ-fed mice in comparison to the controls. IL-2 secretion was increased after stimulation in all treatment animals except those fed fine rice bran compared to the controls. TNF α was significantly decreased

after stimulation with LPS in all the the treatment-fed mice, except those on the wheat germ diet. The oxidized glutathione and malondialdehyde levels in mice fed the treatment diets were lower than those of the control mice, while reduced glutathione levels did not change. Catalase activity in leukocytes was increased in the treatment-fed mice, except for the wheat germ-fed mice. This study demonstrated that polyphenols from cereals can also modulate immunity by influencing the immune responses, cytokines, and redox state of immune cells. While the data reported here are interesting, there are some concerns about the comparison of the treatment diets to the control diets. Twenty percent of the control diet (the biscuits made with the standard rodent diet) had 12 vitamins and minerals that were not found in the treatment diets' cereal-containing biscuits. The vitamins and minerals that were present in the treatment biscuits were also at very different concentrations compared to the control biscuits. The cereal-containing biscuits also had 1.5 to 14 times less carbohydrates and contained 75% to nearly 220% of the protein of the standard chow biscuits. It would be most correct to attribute the findings of this research to the cereal fractions rather than just the cereal polyphenols, since the control and treatment diets have some important differences.

Another article reported the effects of curcumin, a spice polyphenol, on the formation of granulomas in a parasite infection in male CD1 albino mice [17]. Mice infected with *Schistosoma mansoni* were treated with 400 mg/kg body weight of curcumin. The curcumin was partitioned into 16 injections, given twice a week for 8 weeks. After 8 weeks, worm and egg burdens were determined, as were number of leukocytes, hepatic granuloma size, hepatic enzyme activities, cytokine levels liver

fibrosis level, and antibodies against the parasite. The researchers found that the body weights of the infected, treated (IT) mice were similar to non-infected, untreated controls (NU) and non-infected, treated (NT) controls and that the infected, untreated (IU) mice had a significantly lower body weight compared to the others. Liver and spleen weights of the IT group were lower than the IU group, but still higher than either NU or NT controls. The overall white blood cell counts were similar across all groups. Monocytes saw no change across groups, but neutrophils were much higher in both treatment groups compared to the controls. Eosinophils were higher in the IU group compared to the NU and NT controls and the IT mice. Lymphocytes were lowest in the IU mice and slightly higher in the IT mice compared to both control groups. The IT mice had 44% fewer parasites, on average, compared to the IU group. They also had nearly 31% fewer eggs per gram of liver and intestinal tissue compared to their untreated counterparts. Hepatic liver granuloma volume and hepatic collagen were decreased by 79% and 38.6%, respectively, the IT mice versus the IU mice. Alanine aminotransferase (ALT) activity was lower in the IT mice compared to the NU controls, but similar to the NT control. IU ALT activity was lower than the IT mice. Aspartate aminotransferase (AST) activity in the IT group was similar to the NU controls, but lower than the NT controls. AST activity was further depressed in the IU mice compared to the IT mice. Gamma-glutamyl transferase activity in the IT group was similar to both controls. It was lowered in the IU group. Lactate dehydrogenase (LDH) activity was similar between the NU controls and the IT mice. However, the IT mice LDH activity was decreased compared to the NT controls. IU and IT mice had similar LDH activity, but the activity of the IU mice was still lower than both controls. Catalase activity was

high in the IT group compared to all other groups. Serum IL-10 was high in both treatment groups compared to the controls, while IL-12 was only high in the IU mice. TNF α was highest in the IU group, but still elevated to some degree in the IT group compared to controls. IL-4 was not detected in the sera of any mice.

Immunoglobulin G (IgG) and IgG1 levels against soluble worm antigen and soluble egg antigen were increased in the IT group compared to the IU group. IgG2a and immunoglobulin M levels against both antigens were similar in both treatment groups. This research illustrated that curcumin, a polyphenolic compound, can reduce the damage of an inflammatory response to a parasitic infestation, but can also aid in enhancing the efficacy of the immune response by reducing the parasitic load possibly due to the increased antibody levels.

A search of the PubMed database using the terms “polyphenols” and “immun*” provided only one human study result. In this study [18], 7 male and 9 female participants between 20 and 22 years of age were placed on low polyphenol diets or a diet containing 200 g of purple sweet potato leaves per day. All participants were given a control low polyphenol diet for one week before alternating with two weeks of the low polyphenol diet again or the treatment diet containing purple sweet potato leaves. Both diets had similar β -carotene levels. Between the control diet and the treatment diet, participants ate the control diet for one week as a washout period. The urine polyphenol content of participants was measured after each intervention diet. Plasma β -carotene, lymphocyte proliferation, cytokine levels, NK cell activity, and salivary immunoglobulin A (IgA) were measured as well. Plasma β -carotene increased in both diets compared to baseline measurements, while plasma polyphenol levels decreased

in the control diet and remained the same as baseline in the purple sweet potato leaves diet. However, urinary polyphenol levels were decreased in the control diet and increased in the treatment diet. Lymphocyte proliferation after stimulation with Concanavalin A was decreased compared to the baseline while on the control diet, but increased during the second week on the treatment diet. The levels of IL-2 and IL-4 secreted by peripheral blood mononuclear cells were increased only in the treatment diet compared to baseline. NK cell activity was decreased in the second week of the control diet and increased in the second week of the treatment diet compared to baseline. Salivary IgA decreased in the control group, but normalized on the treatment diet. The results are intriguing, especially because it is a human study. However, it had a small number of participants and certainly needs validation. It could also benefit from the measurement of different immune markers and the use of a purified polyphenol supplement instead of ingestion of whole plant parts. The changes seen in the treatment group may be due to the polyphenols of the purple sweet potato leaf, but they also could be due to other compounds in the leaves.

Another human study looks at immunity in more detail, but has a more general focus on phytochemicals in fruits and vegetables instead of only polyphenols [19]. In this study law students were given a commercially available fruit and vegetable concentrate supplement for 11 weeks. T-cell counts, cytokine production, lymphocyte DNA damage, blood antioxidant, vitamin C and carotenoid levels and reported illnesses were assessed. The number of $\gamma\delta$ T-cells in the blood increased and lymphocyte DNA damage decreased in individuals taking the supplement compared to those taking the placebo. Vitamin C and carotenoid levels and oxygen radical absorbance capacity

(ORAC) of the blood also increased compared to baseline. IFN- γ levels decreased in stimulated cells in those taking the supplement compared to the placebo, while other cytokines measured did not change between the two treatment groups. Overall, these results show that compounds in fruits and vegetables administered in capsule form can increase important immune parameters while still encouraging an anti-inflammatory state. However, in this study, these results cannot be solely attributed to polyphenols.

Cranberry

Cranberries and their effect on urinary tract infections have been studied extensively [20-25]. They've also been implicated in variations in cholesterol metabolism [26,27] and as anti-proliferative agents of various human cancer cell lines [28,29].

Cranberry and Periodontal Disease

An emerging area of cranberry research focuses on how cranberry may affect the immune system. Most research in this field deals with the anti-inflammatory capabilities of cranberry and its components in relation to gingival fibroblasts and macrophages [30-33].

In 2006, Bodet et al. [33] published the first research to examine the anti-inflammatory role of cranberry proanthocyanidins on immune cells stimulated with an inflammation-inducing compound, like LPS. The researchers used the U937 leukemia cell line as a model of macrophage immune cells. The cells were differentiated to macrophage-like cells using PMA, incubated with 10 to 50 $\mu\text{g}/\text{mL}$ of a proanthocyanidin-rich cranberry fraction, lyophilized juice fraction, or epigallocatechin (EGCG) for 2 hours and then incubated with LPS for 24 hours before being assayed. Cell viability and the production of IL-1 β , IL-6, IL-8, TNF α and regulated upon activation

normal T-cell expressed and secreted (RANTES) were measured. No cytotoxic effects were seen in any of the test compounds at the various concentrations used. TNF α and IL-6 levels were significantly decreased in the 25 μ g/mL and 50 μ g/mL concentration of the proanthocyanidin fraction and in the 10 μ g/mL concentration of EGCG.

Interestingly, IL-1 β levels increased beyond the control in the LPS stimulated cells incubated with 10 μ g/mL proanthocyanidin fraction. However, at 50 μ g/mL the IL-1 β levels were similar to EGCG, the positive control. Only the proanthocyanidin fraction significantly decreased the IL-8 levels when cells were stimulated with LPS. RANTES was significantly decreased in all three of the proanthocyanidin concentrations and with the 10 μ g/mL EGCG. The lyophilized cranberry juice fraction had no significant effects on any of the cytokines that were tested.

Another study from this same group [34] also examined the ability of cranberry proanthocyanidins to reduce the negative effects of bacterial LPS on gingival fibroblasts. These fibroblasts are sensitive to LPS and mount an inflammatory response to it that can damage healthy tissue when the response becomes chronic. In this study, the fibroblasts incubated with the proanthocyanidins secreted less IL6, IL8, and prostaglandin E2 and had lower cyclooxygenase II expression when the cells were treated with LPS. Seeram et al. [35] showed that cranberry and other berry anthocyanins also inhibited cyclooxygenase II activity. The Bodet group has gone on to show that cranberry proanthocyanidins reduce the production and secretion of matrix metalloproteinase-(MMP) 3 and MMP-9 in macrophages, MMP-3 in gingival fibroblasts, as well as, reducing the activity of these two proteins and elastase in cells stimulated with LPS from periodontal pathogens [32]. The proanthocyanidins also decreased the

expression of intracellular signaling molecules that were up-regulated in fibroblasts treated with LPS.

Expanding on this MMP data, another group of researchers studied how A-type proanthocyanidins affect inflammatory markers [30]. MMP-1, -3, -7, -8, -9, and -13 levels were shown to significantly decrease in cells treated with these A-type proanthocyanidins for two hours and then incubated in LPS for 24 hours compared to cells that had no cranberry treatment. The NF- κ B DNA binding activity was greatly reduced in macrophages treated with the fractions compared to those only having the LPS stimulation. Four protein kinases that were up-regulated in the LPS control had significantly reduced levels when treated with the proanthocyanidins from cranberry.

A recent study [31] showed that cranberry proanthocyanidins had a protective effect on macrophages that were exposed to bacterial cell wall components that normally significantly reduce cell viability. U937 cells were differentiated into macrophage-like cells using PMA. A human oral epithelial cell line, GSM-K, was also used in this experiment. Cell wall from was prepared from *Peptostreptococcus micros* HG1251, a dental pathogen, and used to treat the differentiated U937 cells and oral epithelial cells. Cells were treated with 0, 5, 10, 25, 50 or 100 μ g/mL of cranberry fraction for 2 hours and then cultured with the cell wall at 20 μ g/mL for 24 hours. Cell viability was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction. A 50 μ g/mL concentration of the cranberry fraction resulted in a significant reduction of cell death in the U937 and GSM-K cells.

Cranberry and Immunity

While periodontal disease has been in the forefront of cranberry research in regards to immunity, there are other groups that are taking a more global look at

immunity. This section will discuss the body of research done in relation to cranberry and systemic immunity.

Hochman et al. [36] show that a nondialyzable fraction of cranberry juice slows in vitro growth and movement across membranes of murine lymphoma cells. This research group also tested the effect of injected nondialyzable cranberry fractions on mice that had been inoculated with murine lymphoma cells. Eighty percent of the mice that were injected with the lymphoma cells and that did not receive treatment had tumor growth after 60 days. No mice treated with the cranberry fraction had developed tumors after 100 days post-inoculation. Furthermore, these cranberry-fraction-treated mice had produced anti-lymphoma antibodies.

Delehanty and colleagues examined [37] cranberry proanthocyanidins and their impact on TLR4 and the NF- κ B pathway. The researchers investigated proanthocyanidins from red grape, cranberry juices, and black tea and their ability to bind LPS, alter LPS endocytosis, prevent LPS from binding to TLR4, LPS-binding protein (LBP) and cluster designation (CD) 14, and reduce NF- κ B activation. The cranberry (dialyzable and nondialyzable), tea, and grape proanthocyanidins all bound LPS. The highest molecular weight cranberry proanthocyanidins bound LPS most effectively. The average degree of polymerization for this group of proanthocyanidins was 21. Since the fraction of dialyzable cranberry proanthocyanidins bound LPS the most efficiently, it was used exclusively in the rest of the experiments. These high molecular weight proanthocyanidins were shown to modestly reduce the binding of LPS to the cell surface and significantly reduce LPS endocytosis. However, overall endocytosis was not affected when cells were treated with cranberry proanthocyanidins.

The proanthocyanidins did not inhibit the binding of LPS to LBP, but did inhibit the binding of LPS to CD14 at a concentration of 500 nM. Also at that concentration, proanthocyanidins nearly completely inhibited the interaction of LPS and the TLR4/MD-2 complex. The NF- κ B activity was also significantly reduced when cells were incubated with 10 nM high molecular weight proanthocyanidins.

Together these studies discussed above suggest that cranberry proanthocyanidins have anti-inflammatory properties. Each study used different methods, cell types, and proanthocyanidin concentrations during their research. More evidence is necessary to fully understand and validate these findings. The question of whether other cranberry polyphenols, like anthocyanins, may have similar effects is also raised by this research.

HL-60 Cells

HL-60 cells are a human promyelocytic leukemia cell line. The cells were originally from a 36-year-old female with acute promyelocytic leukemia [38]. Approximately 5% to 10% of cells in culture will spontaneously differentiate, but cells can be pushed into differentiation toward macrophage- or neutrophil-like cells using various chemicals. HL-60 cells are frequently used in research as models for both of these cell types [39-45]. It is advantageous to use a continuous cell line due to their ability for continuous growth, their relative hardiness, and their replacement of human donors every time cells are needed.

In my study, HL-60 cells were differentiated to neutrophil-like cells using all-*trans* retinoic acid (ATRA). Breitman et al. first discussed the granulocytic differentiation of this cell line in the presence of ATRA in 1980 [46]. While this discovery first excited medical doctors and cancer researchers, it has become important to the study of immunity due to the widespread use of the HL-60 cell line.

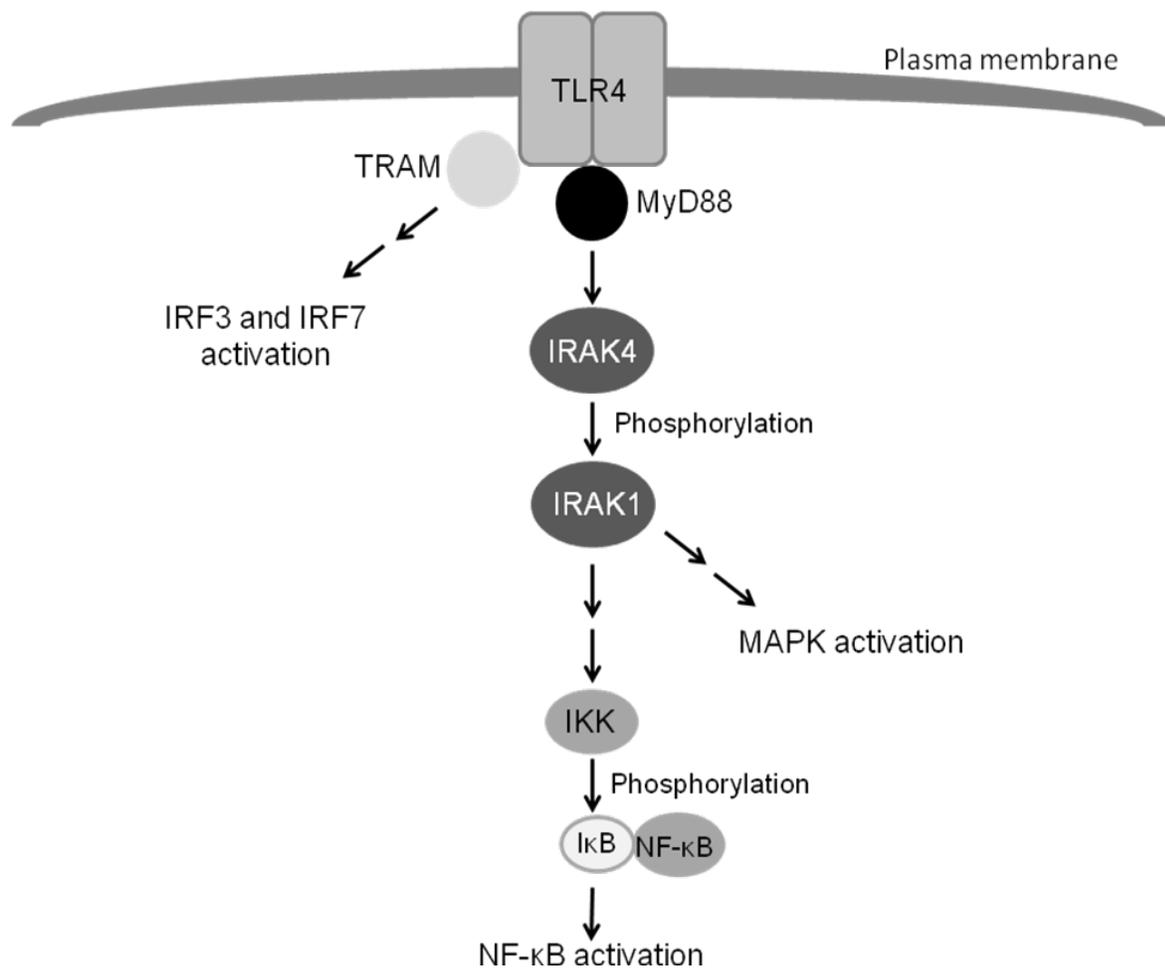


Figure1-1. TLR4 pathway.

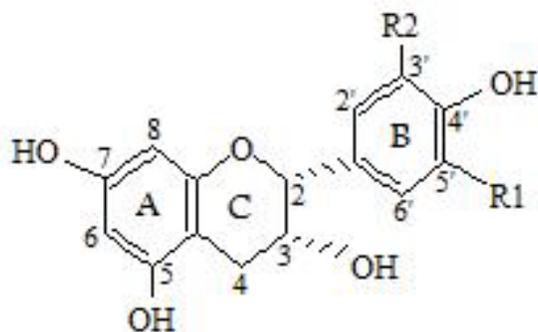


Figure 1-2. Basic structure of a flavonoid.

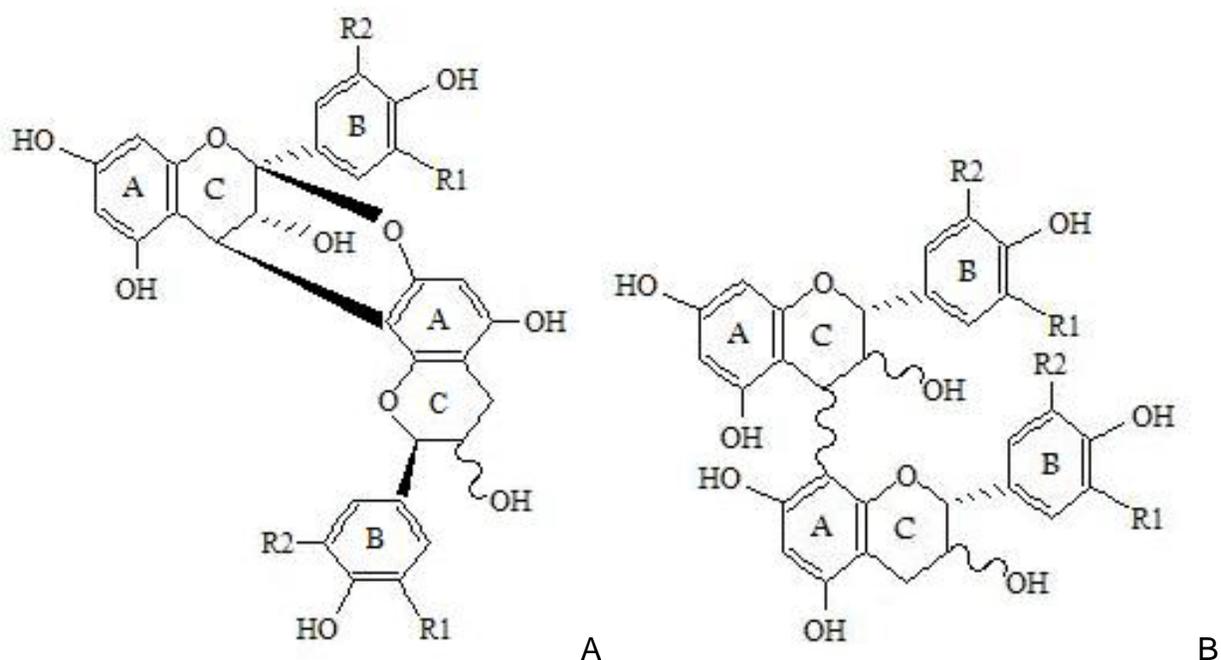


Figure 1-3. Proanthocyanidin structure and linkage. A) Structure of two monomers of a procyanidin with an A linkage between the 2-position of the C ring of one monomer and the hydroxyl group on the 7-position of the A ring of the second monomer and a second bond between the 4-position of the C ring of one monomer and the 8-position of the A ring of the second monomer [7]. The second bond of the A linkage can also be between the 4-position of the C ring of one monomer and the 6-position of the A ring of a second monomer. (B) Structure of two monomers of a proanthocyanidin linked with a B linkage between the 4-position of the C ring of one monomer and the 8-position of the A ring of the second monomer [7].

CHAPTER 2 ANTIOXIDANT ACTIVITY OF CRANBERRY POLYPHENOLS, INCLUDING ANTHOCYANINS AND PROANTHOCYANIDINS

Introduction

Two methods were used to determine the antioxidant capacity of six cranberry fractions. These included the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) methods. Both methods examine the ability to quench different types of radicals and, so, used together they are advantageous in exploring a compound's antioxidant status.

DPPH, the chemical, is a stable nitrogen free radical [47]. The DPPH assay measures the efficiency with which another chemical can subdue this radical. DPPH is deep purple in color when dissolved in methanol or ethanol, with the addition of a strong antioxidant, the color of the solution changes from purple to yellow. This change is measured spectrophotometrically and compared to a standard (e.g., ascorbic acid) at known concentrations. The procedure below used 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, also known as Trolox, for the standard. Trolox is analogous to vitamin E and is water soluble.

ORAC is commonly used in industry and research to quantify oxygen radical absorbance capacity. This assay examines the ability of a compounds to quench a peroxy radical. It was first reported by Cao and colleagues in 1993 [48]. In this assay, 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) produces peroxy radicals. Fluorescein loses fluorescence when it is oxidized by the peroxy radical. If antioxidants are present they prevent the oxidation of fluorescein and, therefore, the loss of fluorescence. The change in fluorescence can be measured with a plate reader and compared to a standard curve.

Materials and Methods

DPPH

Six cranberry fractions were fractionated and provided by Ocean Spray Cranberries, Inc. (Lakeville Mass.). Table 2-1 presents what part of the cranberry each fraction came from and its major constituents.

Fractions A, C, and F are soluble in methanol. They were dissolved in methanol (Fisher Scientific, Fair Lawn, NJ) and compared with a Trolox standard curve (Fluka, Switzerland) in methanol. Fraction B and D are water soluble and these were compared with a Trolox standard curve in water. Fraction E is soluble in water and methanol. It was dissolved in water and compared with the Trolox standard curve in water along with Fractions B and D. The final concentrations of Trolox were: 40 μM , 30 μM , 15 μM , 5 μM , 0.5 μM , and 0 μM .

Fractions were incubated for 30 minutes in the dark at concentrations between 1 and 5 $\mu\text{g/mL}$ with a 0.216 mM final concentration of DPPH (Sigma, St. Louis, MO). Following incubation, fractions along with their standards were plated in quadruplicate in a 96-well plate. They were read at 517 nm using a SpectraMax 340PC 384 (Molecular Devices, Sunnyvale, Calif.) plate reader. Samples were compared to the standard curve to determine their Trolox equivalents (in $\mu\text{mol/mg}$ sample). DPPH was run 3 times for Fractions A, C, D, E, and F and twice for B (due to the limited quantity of fraction B available).

A one-way analysis of variance (ANOVA) (SigmaStat, Version 9, Systat, San Jose, CA) was used to test for significant differences among the means of the \log_{10} transformed data due to a failed normality test and an unequal variance. To test for which means differed, a Tukey's post hoc all-pairwise analysis was used.

ORAC

Fractions A, B, C, D, E, and F were dissolved in an acetone/water/acetic acid (Fisher Scientific) (70%: 29.5%:0.5%, v/v/v) extraction solvent at a concentration of 1 mg of fraction per mL extraction solvent. The samples were sonicated using a Microson sonicator (Misonix, Farmingdale, NY) for 3 short bursts of 5 seconds and all fractions went into solution. The final dilutions of each fraction tested were between 500 and 1700 ng/mL.

The samples were compared to a Trolox standard curve using the following final concentrations of Trolox to make the standard curve: 3.125 μ M, 1.563 μ M, 0.781 μ M, 0.391 μ M, and 0.25 μ M. Each sample and standard were plated in a black Costar plate with a clear, flat bottom (Corning, Corning, NY) with 22.3 nM concentration of fluorescein (Sigma) and allowed to mix and incubate for 10 minutes at 37°C in a SpectraMax Gemini XPS (Molecular Devices) fluorescent plate reader. Immediately after the incubation, AAPH (Wako, Richmond, VA) was added to each well, except for the control wells, at a final concentration of 19 mM and the plate was read. The plate reader was set at 485 nm for excitation and 530 nm for emission with 76 readings taken in 40 minutes. Three independent experiments were conducted.

A one-way ANOVA was used to test for significant differences among the means of the \log_{10} transformed data due to a failed normality test and an unequal variance. To test for which means differed, a Tukey's post hoc all-pairwise analysis was used.

Results

DPPH

Fractions A and F showed the highest antioxidant capacity in the DPPH assay, which examines an electron transfer reaction involving DPPH [49]. Fraction D had the

lowest antioxidant activity of all fractions tested. The P-value for the difference between Fractions A and E is 0.038, while the P-value for the difference between Fractions B and E is 0.001. All other significant differences have a P-value of less than 0.001. The letters above the bars in Figure 2-1 denote significance. Fractions with the same letter are not statistically different from one another.

ORAC

In the ORAC assay, which involves a hydrogen transfer as part of the radical quenching [49], Fractions E and F showed the greatest antioxidant activity. Fraction B showed the lowest amount of activity. All differences between the fractions had a P-value less than 0.001. The letters above the bars in Figure 2-2 denote significance. Fractions with the same letter are not statistically different from one another.

Discussion

Fractions A and F had the greatest ability to quench the nitrogen radical in the DPPH assay, while Fractions E and F were best at scavenging the peroxy radicals in ORAC. Fractions A and F are both enriched in cranberry proanthocyanidins. However, Fraction A contains proanthocyanidins from the presscake, while Fraction F's are from the juice. These two fractions have different DPPH and ORAC results, likely because the overall characteristics of the proanthocyanidins in these two components are different. The characteristics and amounts of polyphenols, in general, can vary between plants, growing seasons, and in response to environmental conditions. They also vary between different parts of the same plant.

Unpublished research performed in Dr. Susan Percival's lab using peripheral blood mononuclear cells have suggested that the cranberry polyphenol fractions that have the most antioxidant activity tended to have more activity in cell culture

experiments. The findings from these previous experiments were used to select Fractions A, E, and F for further study.

Table 2-1. Cranberry fraction origins and constituents

Fraction	Major Component	Portion of cranberry that was fractionated
A	Proanthocyanidins	Presscake
B	Anthocyanidins	Presscake
C	Other phenolics	Presscake
D	Whole presscake	Presscake
E	Polyphenols	Juice
F	Proanthocyanidins	Juice

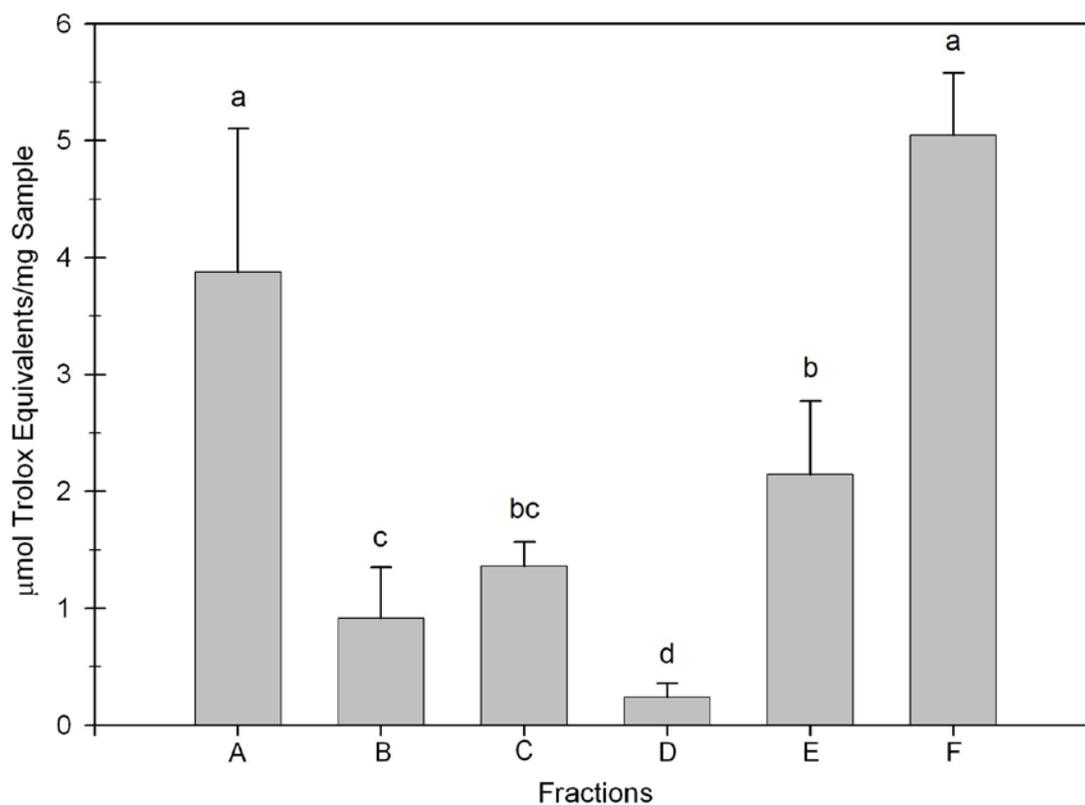


Figure 2-1. DPPH results for cranberry Fractions A through F. The bars represent the mean $\mu\text{mol Trolox equivalents per milligram sample}$ and are shown with their standard deviation for 3 independent experiments (2 independent experiments for Fraction B) for each fraction. Bars with the same letter above are not statistically different from one another.

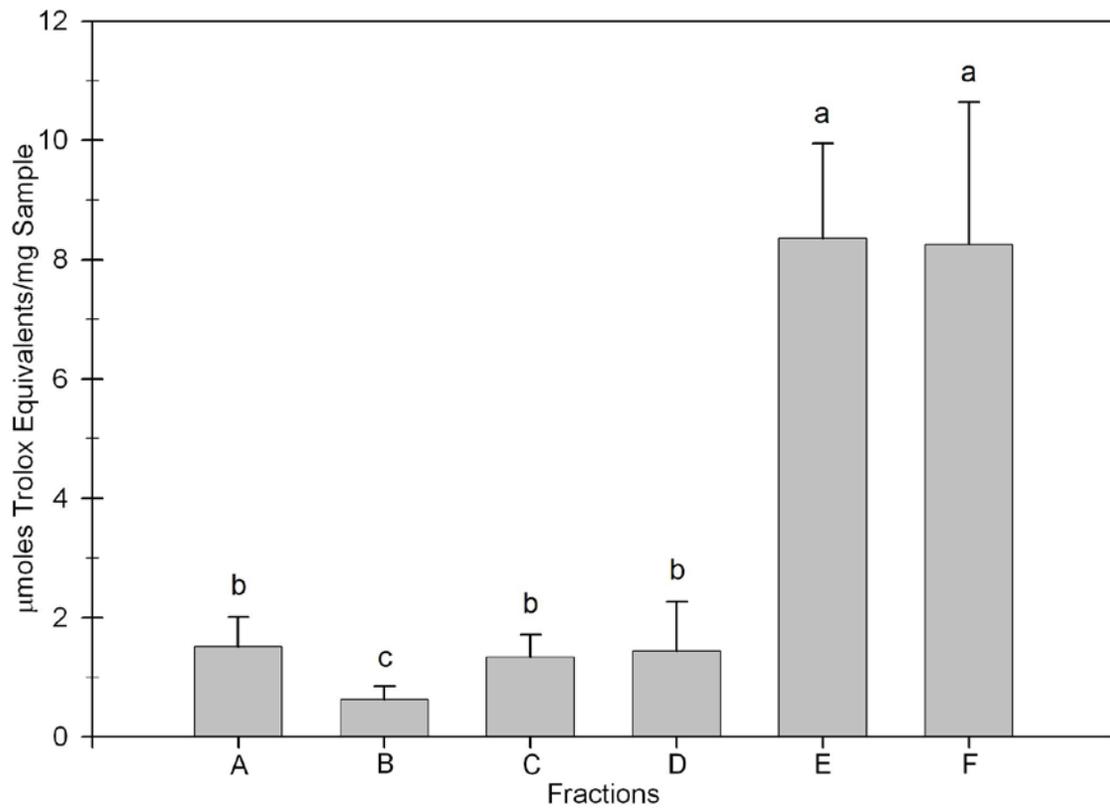


Figure 2-2. ORAC results for cranberry Fractions A through F. The bars represent the mean μmol Trolox equivalents per milligram sample and are shown with their standard deviation for 3 independent experiments for each fraction. Bars with the same letter above are not statistically different from one another.

CHAPTER 3 PROLIFERATION AND DIFFERENTIATION OF HL-60 CELLS AFTER INCUBATION WITH CRANBERRY FRACTIONS

Introduction

It was necessary to determine the highest concentration of each cranberry fraction that affected the cells without causing cell death. In metabolically active cells, cells having functional mitochondria, 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) is taken up and reduced [50]. The metabolism of MTT changes the color of the chemical from yellow to dark purple. This color change can be measured by a spectrophotometer to determine the extent of cell death. This assay does not distinguish between the routes of cell death (i.e. apoptosis versus necrosis), but does offer a quick way to compare cellular growth between treatments.

To determine if differentiation has occurred in cells treated with cranberry fractions the production of superoxide anion in response to a stimulus was measured by the respiratory burst assay. Superoxide anion is usually produced by mature immune cells in response to a pathogen or stimulus, like phorbol myristic acid (PMA). Superoxide anion reduces cytochrome C and this can be measured spectrophotometrically [51]. Immature or undifferentiated immune cells, like promyelocytes, do not secrete superoxide anion in response to a pathogen or stimuli. This kind of killing activity is one of the hallmarks of an immune response in mature immune cells.

Materials and Methods

HL-60 Cell Culture

HL-60 cells (American Type Culture Collection, Manassas, VA) were grown in Iscove's Modified Dulbecco's media (IMDM) with L-glutamine and HEPES (Lonza, Walkersville, MD) and 10% fetal bovine serum (Cellgro, Mediatech, Inc., Herndon, VA),

100 IU/mL penicillin (Cellgro), 100 µg/mL streptomycin (Cellgro), 0.25 µg/mL amphotericin B (Cellgro), and 50 µg/mL gentamicin (Gibco, Invitrogen Corp., Grand Island, NY). Cells were kept between concentrations of 1.5×10^5 cells/mL and 1×10^6 cells/mL. The true passage number was unknown. When they were received from ATCC their passage was assumed to be one. The viability was always equal to or greater than 94% unless otherwise stated.

Proliferation Assay

The proliferation assay, also called the MTT assay, was adapted from the work of Mosman [52]. HL-60 cells were incubated in a particular concentration of cranberry fraction that were dissolved in dimethyl sulfoxide (DMSO) (Sigma) and IMDM media for 48 hours. Each fraction was tested at several concentrations between 0.1 and 1000 µg/mL. At the end of incubation, a final concentration of 238 µg/mL of MTT dissolved in phosphate buffered saline was added. Cells and MTT were allowed to incubate for four hours. Then 100 µL of a 0.04 normal acid isopropanol solution was added and the wells were mixed thoroughly for 5 minutes at room temperature. The acid isopropanol aided in dissolving the blue formazan crystals that had formed. The plate was read at 570 nm using a SpectraMax 340PC 384 and the reference, read at 630 nm, was subtracted from that. Two independent experiments were conducted

Respiratory Burst Assay

HL-60 cells at 4×10^5 cells/mL were incubated in a final concentration of 75 µg/mL of one of the 6 cranberry fractions for 48 hours at 37°C and 5% CO₂. At the end of incubation, cells were washed with PBS twice. Cells were then counted and cytotoxicity was assessed via the trypan blue exclusion dye method. Cells were resuspended in Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium (Cellgro)

with glucose (1 g/L) (Sigma) at a concentration of 1×10^7 cells/mL. Plate cells in a clear 96-well plate with flat bottomed wells along with 100 μ L of DPBS, 58.8 ng/mL of PMA (Sigma), and 1.1 mg/mL of ferricytochrome C from horse heart (Sigma). The plate was read immediately on a SpectraMax 340PC 384 plate reader at 550 nm, from which the absorption at 490 nm was subtracted, for 10 minutes with a read interval of 30 seconds. This procedure was adapted from Babior et al. [51]. Three independent experiments were performed.

A one-way analysis of variance (ANOVA) was used to test for significant differences among the means of the viability data. To test for which means differed, a Student-Newman-Keuls post hoc all-pairwise analysis was used. A Kruskal-Wallis one-way ANOVA was used to test for significant differences among the ranks of the respiratory burst data due to a failed normality test on the means. No post hoc analyses were used to interpret these data.

Results

Proliferation

The MTT data did not show a dose dependent response in the HL-60 cells to varying concentrations of the cranberry fractions. It appeared as though the color of the fractions may have given misleading data (data not shown) due to its absorbance being near the absorbance of the formazan crystals produced in the MTT assay. Therefore, conclusions cannot be made based on this data.

Respiratory Burst

There was no difference (P-value = 0.190) between any of the fractions or the control in the rate of cytochrome C reduction, as shown in Figure 3-1. The rate at which superoxide anion was produced, secreted, and allowed to reduce cytochrome C did not

vary significantly between any of the fraction-treated, PMA-stimulated cells and the control cells that were only stimulated with PMA. The viability of the cells treated with the cranberry fractions was assessed before cells were used in the respiratory burst assay and are presented in Table 3-1. Only Fraction F resulted in a significant change in viability compared to the untreated control cells. The average viability for cells incubated in Fraction F was 46.4%, while the average viability for untreated cells was 82.9%. The P-value for the difference between these two groups was less than 0.001. There was also a trend seen in Fraction A-treated cells for decreasing viability (P-value = 0.079) compared to the controls cells, but it did not approach significance.

Discussion

It was determined that the fractions, even at the low concentrations used, interfered with the spectrophotometric readings necessary for the assay. The lack of reliable results for the MTT assay showed that the fractions have strong absorption near or at an important wavelength. A different method of determining cytotoxicity, the trypan blue dye exclusion method, was used.

Mature immune cells use prooxidants, like superoxide anion, to kill invading pathogens. *In vitro*, these cells can be induced to secrete superoxide anion by exposing them to PMA or some other stimulus. Superoxide release is an effective killing mechanism, but it is also a good example of why chronic inflammation can be so damaging to healthy tissue. The rate of superoxide anion-driven reduction of cytochrome C was used as an indicator of superoxide anion production. This rate was similar between all fraction-treated cells and the controls cells, suggesting that no differentiation occurred during the treatment of HL-60 cells with cranberry fractions.

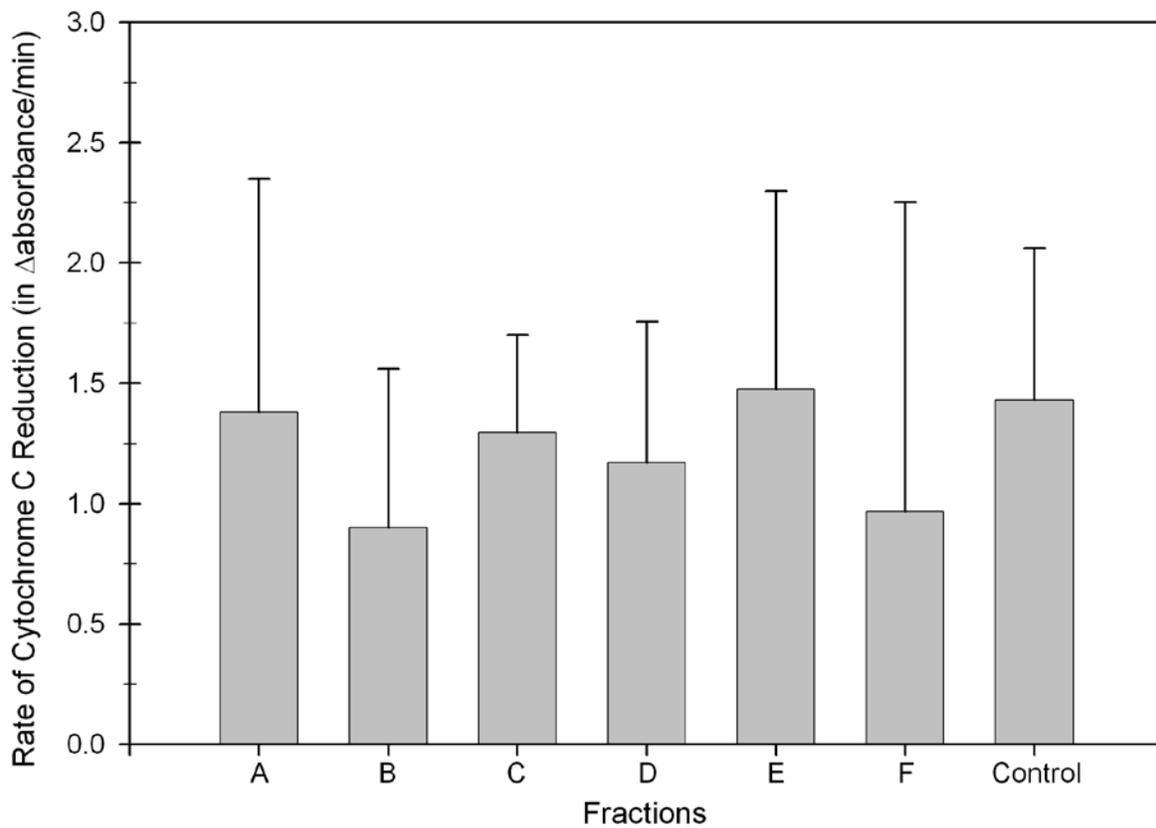


Figure 3-1. Rate of cytochrome C reduction in HL-60 cells treated with 75 µg/mL cranberry fractions

Table 3-1. Cytotoxicity of HL-60 cells in 75 µg/mL cranberry fractions

Fraction	Viability (in percent)
A	67.4 (16.2)
B	80.9 (8.1)
C	80.8 (8.0)
D	82.1 (8.8)
E	76.0 (10.5)
F	46.4 (10.0) *
Control	82.9 (6.5)

The data are presented as mean viability in percent along with the standard deviation of four experiments in parentheses. The asterisk, *, denotes a significant difference compared to the control viability.

CHAPTER 4 CYTOTOXICITY OF CRANBERRY FRACTIONS AND LIPOPOLYSACCHARIDE ON DIFFERENTIATED HL-60 CELLS

Introduction

The next part of the hypothesis was to determine the cranberry fractions' effects on particular protein levels in differentiated cells. Before approaching this task, it was necessary to determine the highest dose of each fraction that differentiated cells can tolerate. The methods and results are discussed in this chapter.

Materials and Methods

HL-60 Cell Differentiation

HL-60 cells were transferred to a separate flask at a concentration of approximately 6×10^5 cells/mL to 8×10^5 cells/mL and incubated for 96 hours in $1 \mu\text{M}$ all-*trans* retinoic acid (ATRA) (Sigma) dissolved in 95% ethanol at 37°C , 5% CO_2 , and humidity as previously reported by Freeman et al. [41]. After the incubation, cells were considered differentiated and ready for experiments. To verify that differentiation occurred, the respiratory burst assay previously mentioned in Chapter 3 was run using the ATRA-treated cells and the same design as discussed in that chapter.

ATRA-treated cells were compared to vehicle-treated control cells. Five independent experiments were conducted. A Mann-Whitney rank sum t-test was used to test for significant differences between the medians due to a failed normality test.

Cytotoxicity

Differentiated HL-60 cells were plated at 5×10^5 cells/mL in a clear, tissue culture treated 12-well plate and incubated for 24 hours at 37°C , 5% CO_2 , and humidity in 250 ng/mL lipopolysaccharide (LPS) (Sigma) and 50 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, or 0.1 $\mu\text{g/mL}$ of one of the fractions which were dissolved in dimethyl sulfoxide (DMSO)

and Iscove's Modified Dulbecco's medium (IMDM) media. Control cells were incubated in only vehicle, excluding DMSO, with no LPS or cranberry fractions present. A second set of cells was incubated with only 250 ng/mL of LPS and no cranberry fractions, but included 0.1% DMSO, the highest amount of DMSO found in the cells incubated with cranberry fractions. At the end of the 24 hour incubation, cell counts and viability were assessed using the trypan blue dye exclusion method. Four independent experiments were conducted.

A one-way analysis of variance (ANOVA) was used to test for significant differences among the means of the data. To test for which means differed, a Student-Newman-Keuls post hoc all-pairwise analysis was used.

Results

HL-60 Cell Differentiation

The superoxide anion-driven reduction of cytochrome C in the ATRA-treated cells was approximately 20 times that of the reduction of cytochrome C seen in the control cells as shown in Figure 4-1. There was a significant difference between the untreated cells and the ATRA-treated cells ($P < 0.001$).

Cytotoxicity

The ANOVA showed that the differences between the data, shown in figure 4-2, was due to more than just chance (P -value = 0.025). However, there were no significant differences found by the pairwise multiple comparison between any of the fractions or the controls. Only one concentration of one fraction, the 50 $\mu\text{g/mL}$ fraction E-treated cells, approached significance (P -value = 0.051) versus the negative LPS, negative DMSO control. The trend was for the cells under this treatment to have a

slightly higher viability compared to cells that were incubated with no DMSO and no LPS. However, this was not statistically significant.

Discussion

HL-60 Cell Differentiation

It is evident that 1 μ M ATRA induces considerable superoxide anion production in HL-60 cells after a 96-hour incubation and stimulation with phorbol myristic acid (PMA). This is classic behavior indicative of mature immune cells. It is also an established fact that prolonged exposure to ATRA induces HL-60 cell differentiation toward the neutrophil-like cells [46,53]. It was determined that the previously described procedure for differentiation was effective and was used in further experiments where differentiated cells were needed.

Cytotoxicity

There was no significant difference in the viabilities of any fractions tested versus the controls. It appeared that the amount of DMSO used to dissolve the cranberry fraction, the concentration of LPS used to stimulate the differentiated cells, or the concentration of any fraction studied had no effect on the viability of the differentiated HL-60 cells. Due to this finding, the concentration of Fraction A, E, and F used in further experiments was 50 μ g/mL. The concentrations of LPS and DMSO used in the cytotoxicity experiment was also the same as those used in the inhibitor of kappa B alpha ($I\kappa B\alpha$), interleukin-1 receptor-associated kinase 4 (IRAK4), and tumor necrosis factor alpha (TNF α) studies.

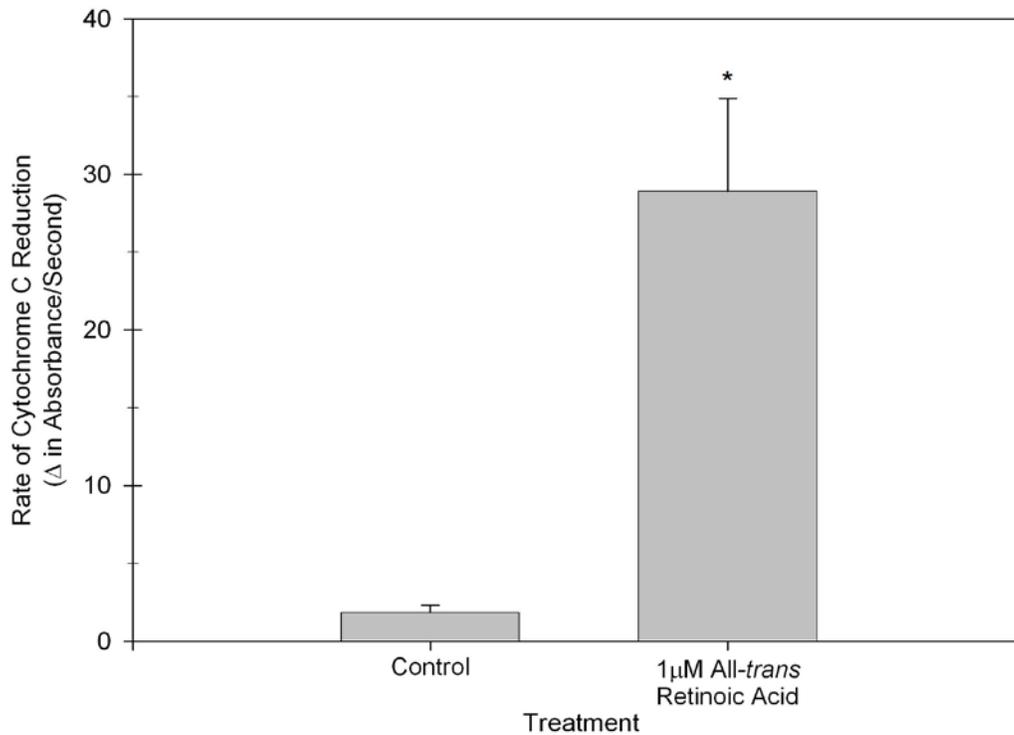


Figure 4-1. Rate of cytochrome c reduction in ATRA-treated cells versus untreated cells. The asterisk, *, denotes a significant difference between the ATRA-treated cells and the control cells.

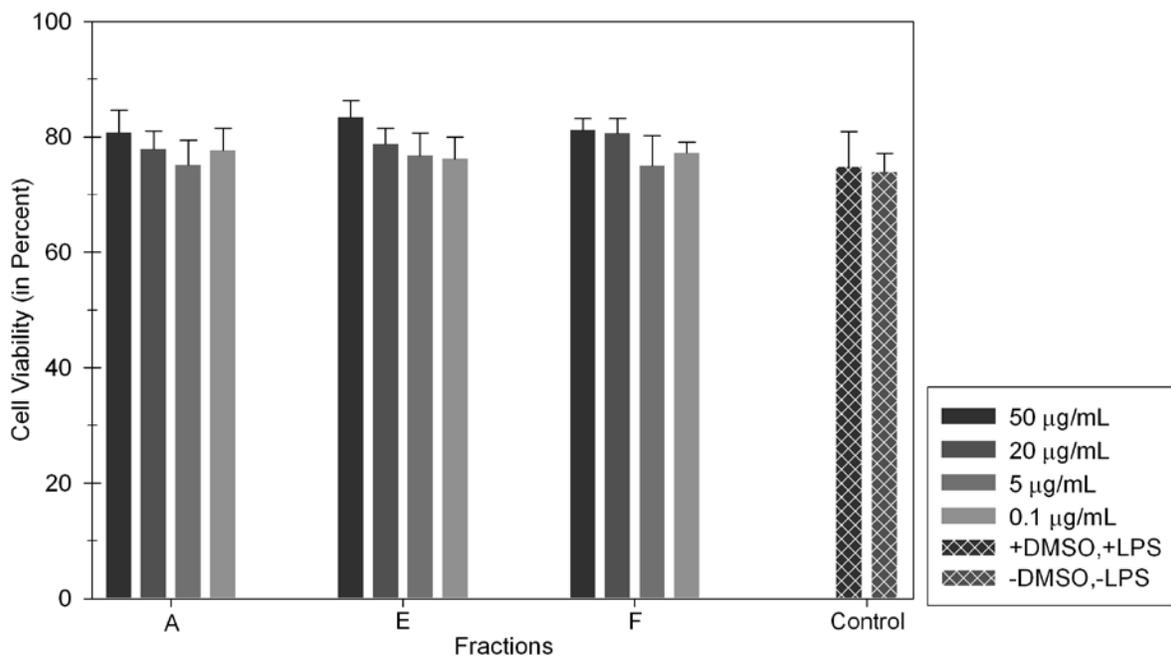


Figure 4-2. Viability of differentiated HL-60 cells treated with various concentrations of six cranberry fractions and LPS.

CHAPTER 5
TOTAL AND PHOSPHORYLATED INHIBITOR OF KAPPA B ALPHA AND
INTERLEUKIN-1 RECEPTOR ASSOCIATED KINASE 4 PROTEIN LEVELS IN HL-60
CELLS AFTER INCUBATION WITH CRANBERRY FRACTIONS AND
LIPOPOLYSACCHARIDE

Introduction

Inhibitor of kappa B (I κ B) is an important regulatory protein in the nuclear factor-kappa B (NF- κ B) pathway. It binds NF- κ B and keeps it from entering the nucleus. This prevents the increased transcription of NF- κ B-inducible inflammatory genes. The first step in the separation of NF- κ B and I κ B is the phosphorylation of I κ B. Once I κ B is phosphorylated, it is marked for ubiquitination and subsequent degradation.

The NF- κ B pathway can be stimulated by various pathogen components and through a variety of mechanisms. The toll-like receptor (TLR) pathway is one that eventually results in the cleavage of NF- κ B and I κ B. Interleukin-1 receptor-associated kinase 4 (IRAK4) is one of many downstream signaling proteins in the TLR pathway. Any changes in its expression have been associated with changes in NF- κ B activation [54-63].

Materials and Methods

Differentiated HL-60 cells were treated with 50 μ g/mL of either Fraction A, E, or F dissolved in dimethyl sulfoxide (DMSO) and media and 250 ng/mL of lipopolysaccharide (LPS) dissolved in media. Control cells were treated with a DMSO and media control solution and 250 ng/mL of LPS or a vehicle-only replacement. In past research, the greatest difference between total and phosphorylated I κ B α (pI κ B α) has been seen 10 to 15 minutes after treatment [57,64]. The 2-hour incubation time chosen for IRAK4 was also based on the procedures of previous research [65]. Fractions were incubated with cells for 10 minutes before collection for I κ B α analysis and for 2 hours before collection

for IRAK4 analysis. Immediately after addition of the fractions, 250 ng/mL of LPS was also added to the cells before incubation began. LPS was also added to cells that had only been treated with a vehicle control. This was the LPS control. A second control, the negative control, had only vehicle added in place of a cranberry fraction and LPS.

After incubation, between 2×10^7 and 3×10^7 cells were collected, washed twice with phosphate buffered saline and resuspended in an aqueous buffer containing protease inhibitors, including 1 mM phenylmethylsulfonyl fluoride (Fisher), 10 μ g/mL pepstatin A (Sigma), 20 μ g/mL leupeptin (Sigma), 10 μ g/mL aprotinin (Sigma), 2 mM ethylenediaminetetraacetic acid (Fisher), and 5 mM dl-dithiothreitol (Sigma). Phosphatase inhibitors, 25 mM sodium fluoride (Fisher), 1 mM sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA), and 10 mM β -glycerophosphate (Sigma), were added to the buffer for experiments pertaining to plkB α . Cells were kept on ice and sonicated for 6-10 second bursts. After sonication, Triton X-100 (Sigma) was added at a final concentration of 1 mM. Samples were, then, centrifuged at 4500 rpm to pellet cellular debris. Supernatant was removed and used for Western blotting. Protein content was determined by the Bradford method. Between 40 and 75 μ g of protein were loaded into each well of the gel. Samples were separate using a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) for one hour at 95 V with a 10% methanol (Fisher), 25 mM Tris (Sigma), 192 mM glycine (Bio-Rad) transfer buffer.

For detection, blots were blocked overnight in 5% non-fat dry milk blocking buffer at 4°C. The primary antibodies were diluted 1:1000 and incubated with blots for 1 hour at room temperature. The plkB α , Ikb α , and IRAK4 antibodies were purchased from Cell

Signaling Technology (Danvers, MA) and glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Imgenex (San Diego, CA). The secondary anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK) was also incubated with the blot under these conditions. Detection solutions were mixed 1:1 and incubated with the blot for one minute and detection occurred immediately after. Films (Fisher) were exposed to blots between 1 and 20 minutes depending on the amount of protein on the blot. Proteins of interest were detected first and, then, the blots were stripped and reprobed for the loading control proteins. IRAK4's loading control was glyceraldehyde-3-phosphate dehydrogenase and I κ B α 's was α -tubulin.

Films were scanned into the SigmaGel software for analysis. Protein bands were compared based on pixel intensities and a ratio of the protein-of-interest to the loading control was obtained for each fraction and controls. To account for differences between gels in the amount of protein loaded into the wells of a gel, the ratio of protein-of-interest to loading control for the LPS control for each blot was set to one arbitrarily. The negative control and fraction-treated samples were then normalized to the LPS control. Four independent experiments were run for total I κ B α and IRAK4 protein, while 3 independent experiments were performed for pI κ B α protein levels.

A one-way analysis of variance (ANOVA) was used to test for significant differences among the means of the pI κ B α data. To test for which means differed, a Student-Newman-Keuls post hoc all-pairwise analysis was used. For the IRAK4 and total I κ B α data a Kruskal-Wallis one-way ANOVA was used to test for significant differences among the ranks of the data due to failed normality tests. To test for which

medians differed, a Dunn's post hoc multiple comparison versus the LPS control analysis was used.

Results

Fraction A- and F-treated cells had a significant increase ($P < 0.05$) in total I κ B α protein levels compared to the LPS control cells as shown in Figure 5-1A. pI κ B α protein levels, illustrated in Figure 5-1B, were significantly lower than the LPS control in Fraction A-treated cells ($P = 0.039$), Fraction F-treated cells ($P = 0.043$), and in the negative control cells ($P = 0.032$). Figure 5-1C shows that IRAK4 protein levels were significantly lower in the Fraction A-treated cells ($P < 0.05$) compared to the LPS control cells.

Discussion

NF- κ B is a transcription factor that acts to increase the transcription of inflammatory genes for growth factors, stress proteins, adhesion molecules, and cytokines and their receptors [66]. It remains bound to I κ B in the cytosol of cells until I κ B's ubiquitination and degradation. The inhibitor of I κ B kinase (IKK) phosphorylates I κ B which signals it for ubiquitination. Once unbound, NF- κ B can move into the nucleus.

Many types of pathogens activate NF- κ B via a multitude of pathways. One of the triggers for NF- κ B is LPS. LPS is recognized by TLR4 and signal transduction through this pathway ends up activating NF- κ B. While an inflammatory response is necessary to mount an attack on invading pathogens, inflammation due to autoimmune disease or excessive or chronic inflammation can have serious negative effects on one's health. For this reason, anything, including plant polyphenols, that can dampen an exaggerated immune response has been of great interest to researchers.

According to the protein data, Fractions A and F have shown the most promise across the board. After a 10-minute incubation with LPS and Fractions A or F, the neutrophil-like cells had a lower amount of $\text{pI}\kappa\text{B}\alpha$ protein and a higher level of total $\text{I}\kappa\text{B}\alpha$ compared to the LPS control. Also, the cells treated with Fraction A and LPS had lower IRAK4 protein levels in comparison to the LPS controls. Taken together this data paints a picture of the ability of the proanthocyanidin-rich cranberry fractions to diminish the inflammatory effect of LPS in neutrophils. It appears that the proanthocyanidin-rich fractions prevent the phosphorylation and degradation of $\text{I}\kappa\text{B}\alpha$, thus it can continue to keep NF- κB in the cytosol of the cell. The lower levels of IRAK4, a signaling protein in the TLR4 pathway, suggest a mechanism by which the proanthocyanidin-rich fractions have their effect.

For these experiments, it is important to note the possibility of the incubation time having an effect on the results. The incubation times were carefully chosen based on published research in order to find times that allow for a large change from baseline. A time course over 12 or 24 hours would be helpful in verifying the changes in protein levels and would give a clearer picture of exactly when these changes occur.

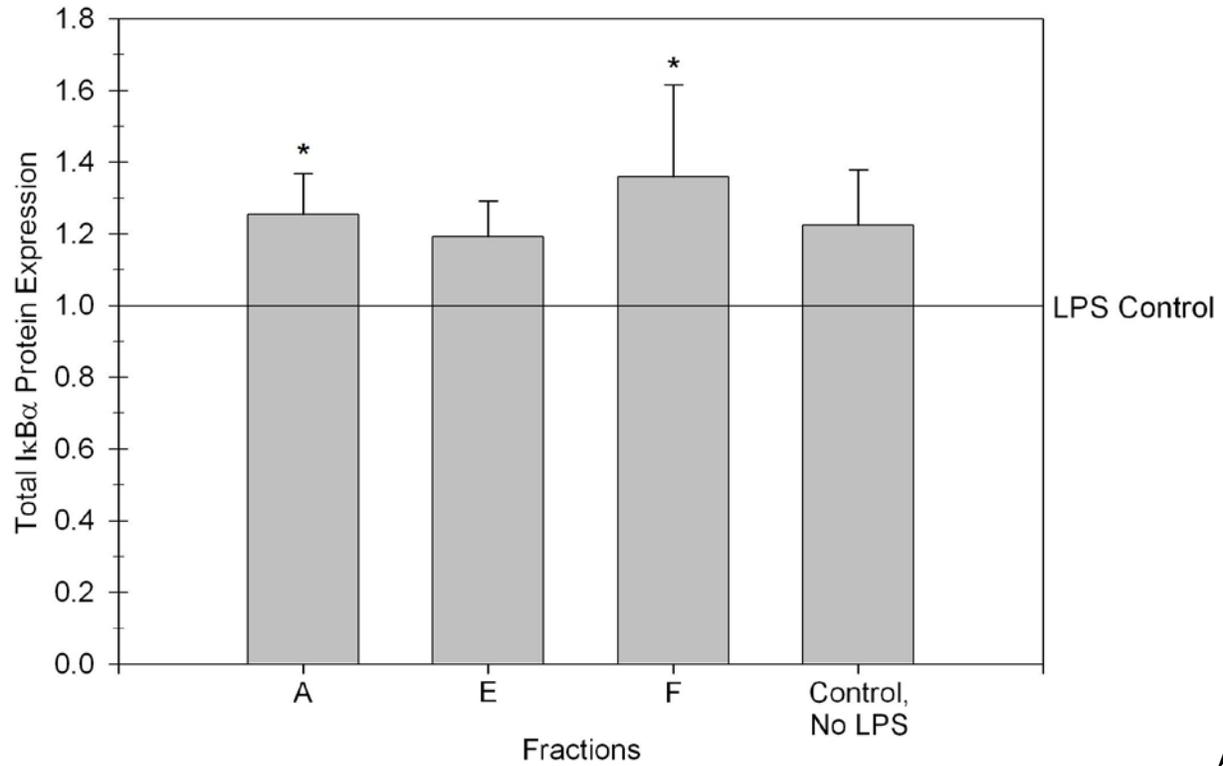
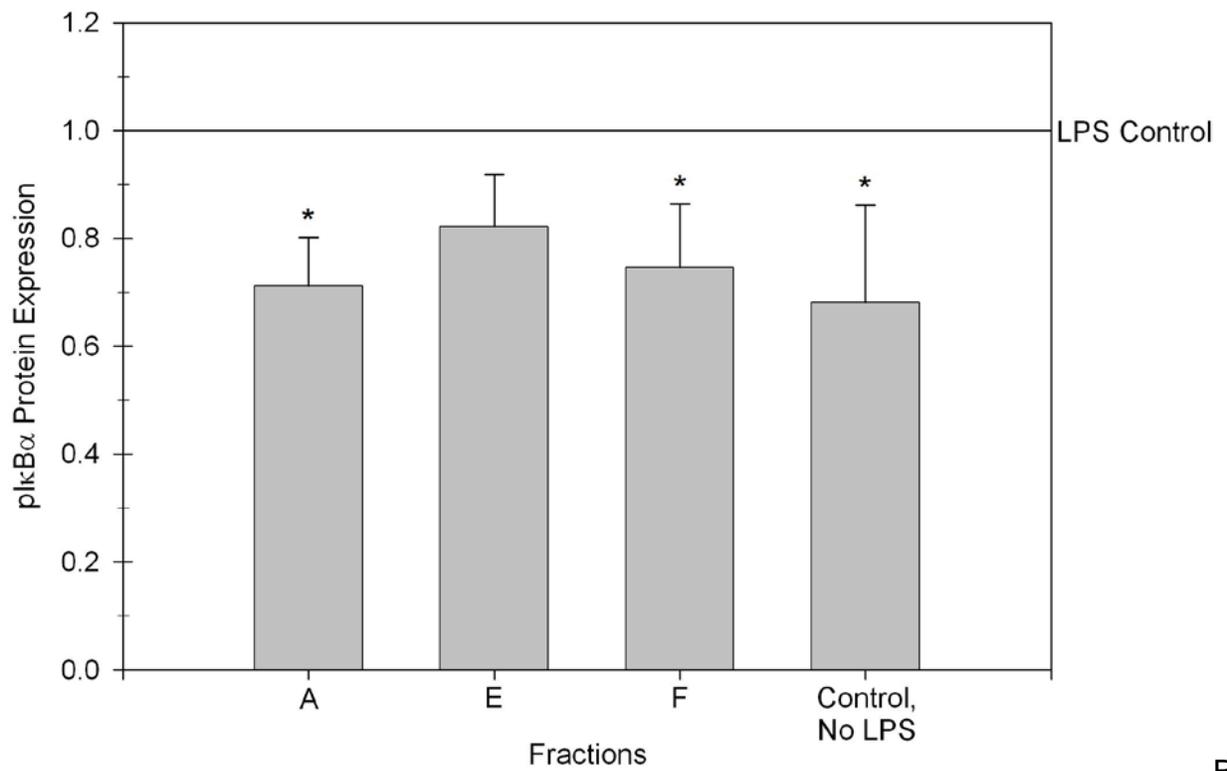
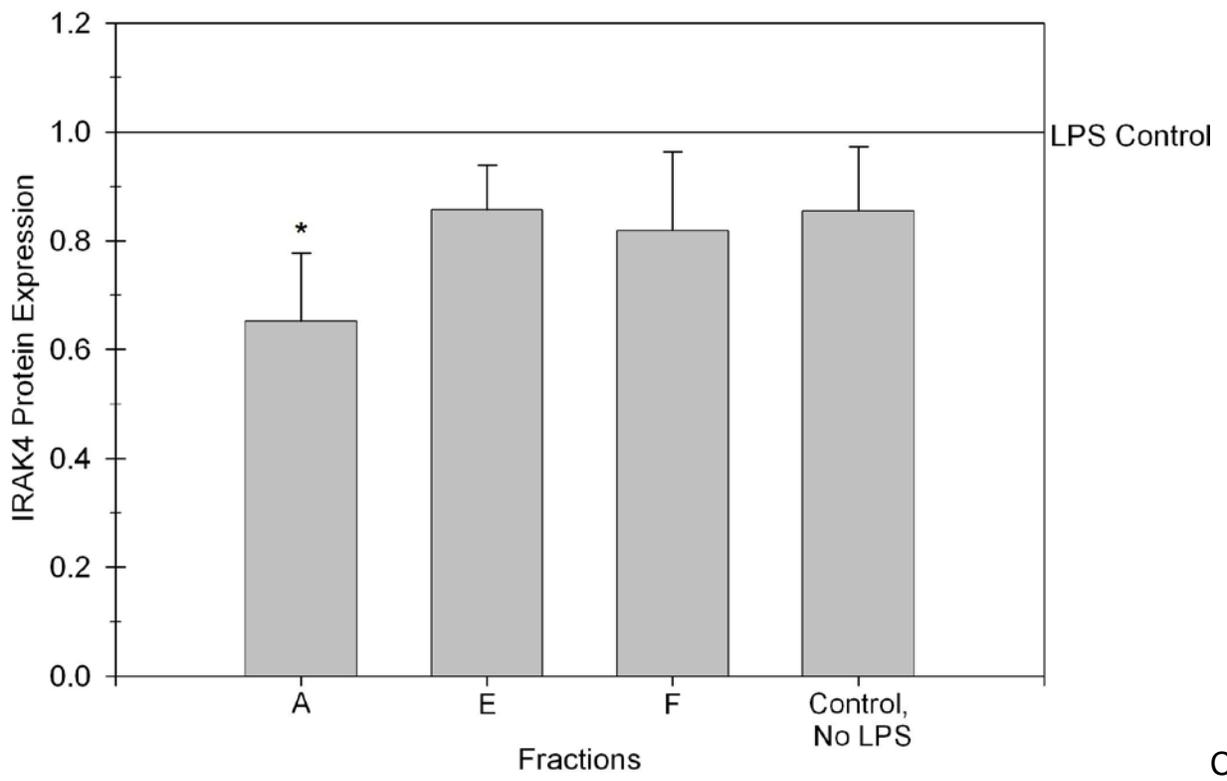


Figure 5-1. Total and pIκBα and IRAK4 protein levels after treatment with cranberry fractions. Cells were treated with 50 μg/mL of Fraction A, E, or F or with a vehicle control. LPS was added to all samples containing fractions and the LPS control. A negative control contained no cranberry fractions and no LPS. Protein expression is in arbitrary units with the LPS control protein expression set to one. A) Total IκBα protein level, B) pIκBα protein levels, C) IRAK4 protein levels. Asterisks, *, above the bars indicate statistical significance in comparison to the LPS control.



B



C

Figure 5-1. Continued

CHAPTER 6 TUMOR NECROSIS FACTOR ALPHA SECRETION INCREASES WITH INCUBATION IN CRANBERRY FRACTIONS

Introduction

One of the hallmarks of nuclear factor-kappa B (NF- κ B) activation is the production and release of inflammatory cytokines. Tumor necrosis factor alpha (TNF α) is one of these cytokines. An enzyme-linked immunosorbent assay (ELISA) assay, described in this chapter, examines the amount of TNF α released by neutrophil-like HL-60 cells after stimulation with LPS and treatment with cranberry polyphenols.

Materials and Methods

HL-60 cells were incubated with 50 μ g/mL of cranberry fraction A, E, or F and 250 ng/mL of lipopolysaccharide (LPS) for 24 hours at 37°C, 5% CO₂, and in a humidified environment. Cells were collected after incubation and centrifuged at 600 times gravity for 10 minutes. The resultant supernatant was used for further analysis of TNF α secretion

A TNF α ELISA kit (eBioscience, San Diego, CA) was used to measure the TNF α in the supernatant. The ELISA procedure provided by the eBioscience kit was followed, but a brief description of the procedure can be found below.

Plates were coated with capture antibody overnight at 4°C. Wells were washed 5 times for one minute per wash using wash buffer. Then, wells were blocked with assay diluents for 1 hour at room temperature. The plate was washed again as previously described. The TNF α standard was plated in the following concentrations: 500 pg/mL, 350 pg/mL, 250 pg/mL, 125 pg/mL, 50 pg/mL, 10 pg/mL, and 4 pg/mL. Samples were plated in triplicate. The plate was incubated overnight at 4°C and, then, washed. Detection antibody at a 1/250 dilution was added to each well and incubated

for 1 hour at room temperature. The plate was washed again. The avidin-horseradish peroxidase enzyme was diluted 1 to 250 and added to the plate for a 30-minute incubation at room temperature. Wells were washed 7 times for one minute per wash and substrate solution was added to each well. The plate was incubated for 15 minutes at room temperature. A 2 normal sulfuric acid stop solution was added to each well and the plate was read at 450 nm and 570 nm on a SpectraMax 340PC 384. The second wavelength was subtracted from the first. Four independent experiments were conducted.

A one-way analysis of variance (ANOVA) was used to test for significant differences among the means. To test for which means differed, a Student-Newman-Keuls post hoc all-pairwise analysis was used.

Results

TNF α levels released by the cells treated with cranberry fractions and LPS, shown in Figure 6-1, were approximately 10 times higher than cells treated with LPS alone. Fraction A- and Fraction F-treated cells had that highest TNF α secretion at approximately 600 pg/mL, while Fraction E-treated cells had slightly less at about 400 pg/mL. The TNF α level in the supernatant of unstimulated differentiated HL-60 cells was not detectable, while the LPS-stimulated cells had an average concentration of approximately 60 pg/mL.

The differences between all fraction-treated cells and the controls all have P-values less than 0.001. The difference between Fraction A-treated cells and Fraction E-treated cells has a P-value of 0.005, while the difference between Fraction F-treated cells and Fraction E-treated cells has a P-value of 0.002.

Discussion

The large increase in TNF α secretion in the fraction treated cells was contrary to what was expected. The experiments involving inhibitor of kappa B alpha (I κ B α) and interleukin-1 receptor-associated kinase 4 (IRAK4) protein levels suggested that signaling through toll-like receptor 4 (TLR4) and NF- κ B activation were similar to unstimulated cells or at least reduced in comparison to the LPS control. However, the TNF α data presented above are suggesting another pathway is stimulated by the cranberry polyphenols, resulting in an increased TNF α production and secretion.

Despite the large body of research that shows plant polyphenols have anti-inflammatory capabilities there are several studies that have found seemingly opposing data [12,67,68]. Wang et al. studied 23 polyphenols, including phenolic acids, isoflavones, flavonols, and anthocyanins, and berry extracts in relation to their effects on TNF α secretion and nitric oxide production in the RAW 264.7 mouse monocyte/macrophage cell line.[12]. Cells were incubated with 16 to 500 μ M concentrations of various polyphenols or 16 to 500 μ g/mL of berry extracts for 1 hour and then exposed to LPS and IFN- γ for 24 hours before measuring TNF α and nitric oxide production. At higher concentrations daidzin, genistin, cyanidin, malvidin, pelargonidin, peonidin, crude blackberry extract, and blueberry concentrate all increased TNF α levels beyond the LPS/IFN- γ only control. At most or all concentrations tested the following increased TNF α levels: daidzein, gallic acid, kaempferol, myricetin, delphinidin, cyanidin 3-glucoside, crude Saskatoon berry extract, blackberry concentrate, and black currant concentrate.

Park et al. [67] used the same cell line to study monomeric, dimeric, and trimeric flavonoids and pycnogenol, a maritime pine bark extract. The monomeric and dimeric

flavonoids at 100 µg/mL did not increase TNFα secretion in unstimulated macrophages. However, a trimeric flavonoid and pycnogenol at 100 µg/mL increased TNFα. In interferon-gamma (IFN-γ) stimulated cells, monomeric flavonoids, like catechin, and dimeric flavonoids reduced TNFα secretion at 100 µg/mL. The trimeric flavonoid and pycnogenol, on the other hand, approximately doubled the TNFα secretion compared to the IFN-γ stimulated controls. These experiments also showed that NF-κB-dependent gene expression nearly doubled when cells were incubated with a trimeric flavonoid or pycnogenol and IFN-γ, while the monomeric flavonoids showed expression similar to the unstimulated control and the dimeric flavonoids had slightly reduced expression in comparison to the IFN-γ stimulated control.

A third study showed that quercetin and resveratrol, at a concentration of 0.2 mM and 0.1 mM, respectively, did not inhibit LPS-stimulated p50/65, an NF-κB heterodimer, activation in RAW 264.7 cells [68]. Quercetin did inhibit the activation of the p50/50 homodimer. Resveratrol also increased TNFα secretion in unstimulated cells and in cells treated with LPS.

It is important to note the purity of the treatment in addition to the effects on immune cells. A quercetin or genistin treatment is expected to be relatively pure, while a crude extract of blueberry is likely to be just that. It is possible that compounds other than polyphenols in these crude extracts may be having an effect on the cells. Chemicals within these extracts may inhibit the activity of polyphenols or may synergistically enhance their activity.

While the preponderance of data on polyphenols from cranberry and other plant sources suggests they are generally anti-inflammatory. The nearly 10-fold increase in

TNF α , an inflammatory cytokine, secretion in polyphenol-treated neutrophils found in my study seems to suggest otherwise. The research discussed above presents evidence to corroborate these findings. It is interesting to note that the data reported by Wang et al. [12] and Park et al. [67] was the result of using higher concentrations of polyphenols compared to my study. These two studies used up to 500 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, respectively, while my study used concentrations of 50 $\mu\text{g}/\text{mL}$. The Wang et al. research [12], which found a few polyphenols and berry extracts that reduce TNF α production and several that enhance it, is a fitting illustration of the dichotomy of polyphenols' effects on inflammatory responses in cell culture.

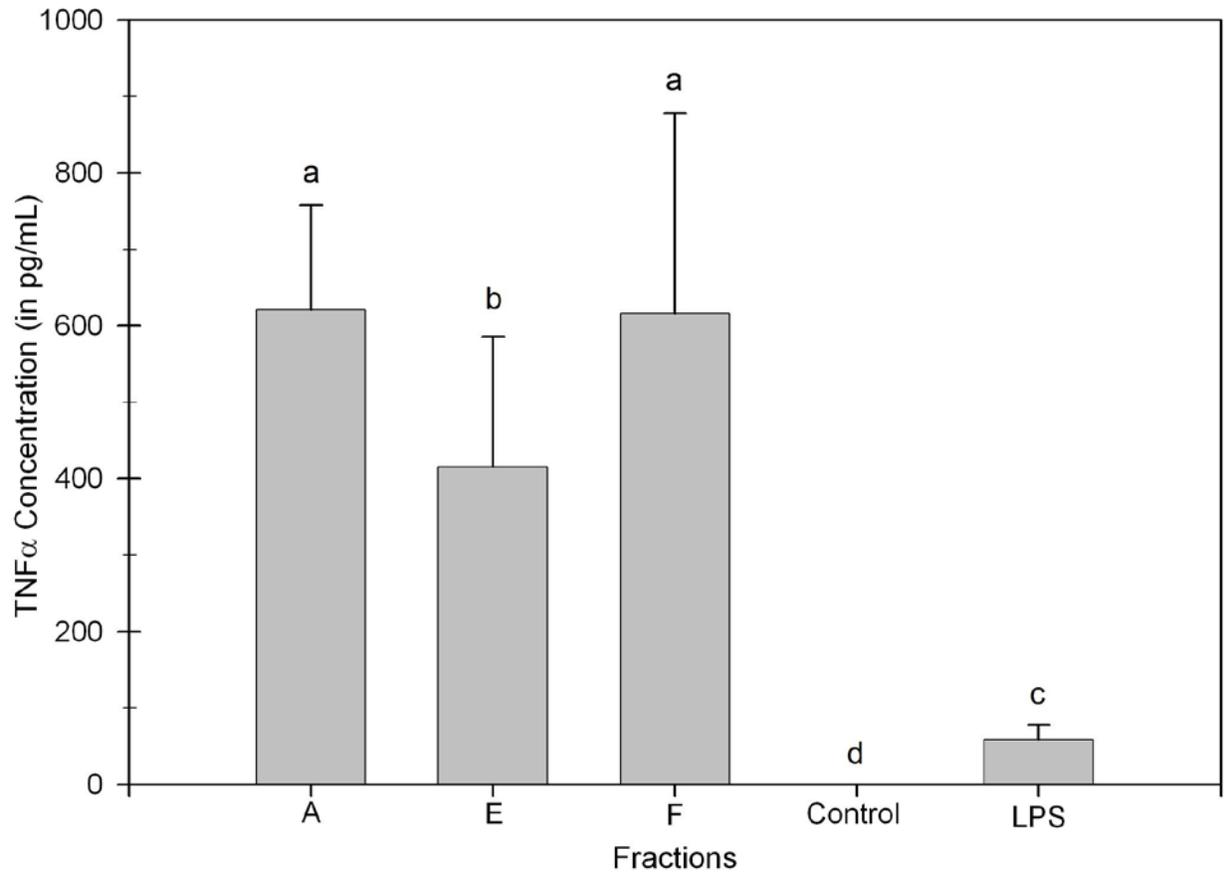


Figure 6-1. TNF α secretion after 24-hour incubation in cranberry fractions and LPS. The thick bars represent the mean of four experiments and the tails represent the standard deviation. Bars with the same letter above are not significantly different.

CHAPTER 7 CONCLUSION

This project has shown that cranberry fractions enriched in presscake proanthocyanidins, Fraction A, and juice proanthocyanidins, Fraction F, have the highest nitrogen radical quenching ability of all of the fractions tested in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The juice polyphenol-rich fraction, Fraction E, and Fraction F are best at quenching the peroxy radical from the oxygen radical absorbance capacity assay (ORAC).

Differentiated HL-60 cells tolerated all fractions up to 50 µg/mL. Lipopolysaccharide (LPS)-stimulated differentiated cells showed reduced phosphorylated inhibitor of kappa B (pIκBα) protein levels when treated with Fraction A and Fraction F. The protein levels of total inhibitor of kappa B alpha (IκBα) were significantly higher in cells treated with these two fractions. Furthermore, interleukin-1 receptor-associated kinase 4 (IRAK4) protein levels were decreased in cells treated with Fraction A and LPS. The decreases in this protein in Fraction F-treated cells are suggestive, but not significant. These data indicates that the proanthocyanidin-rich fractions from cranberry presscake and juice attenuate LPS-induced nuclear factor-kappa B (NF-κB) activation by preventing the phosphorylation and degradation of IκBα. They also suggests that at least one way it accomplishes this is through interrupting the toll-like receptor 4 (TLR4) pathway, as shown by the reduced IRAK4 protein levels in Fraction A-treated cells and the downward trend of IRAK4 protein levels in Fraction F-treated cells.

However, the large increases in secreted tumor necrosis factor alpha (TNFα) seen in fraction-treated cells point to the activation of another pathway by the cranberry

fractions. This is stimulation above that seen by LPS alone. Taken together, this shows that the cranberry polyphenols studied have promising immune modulating abilities. They can prevent mass activation of inflammatory genes, but still allow for targeted inflammatory cytokine secretion in response to a pathogen. This may make them better targets for further research into the treatment of severe or chronic inflammation, like sepsis or autoimmune disease.

My study tested polyphenol-rich cranberry fractions at a concentration of 50 µg/mL in an *in vitro* system. This concentration is approximately 10 to 1000 times higher than previously measured plasma levels of various flavonoids [69]. It is difficult to measure plasma polyphenols that originate from the diet because gut bacteria metabolize them. This modification likely changes their activity in the body and increases the number of metabolites to measure in the blood. However, it is important to note that gut-associated lymphoid tissues sample intestinal contents for pathogens and likely come in contact with concentrations of polyphenols in excess of 50 µg/mL. This may have implications in the way these immune cells react to gastrointestinal (GI) disorders, like inflammatory bowel disease or the manner in which an immune response is mounted to pathogens detected in the GI tract. Furthermore, my study is important as a jumping-off point for further research of these cranberry fractions. It reveals the activities of these fractions as antioxidants and in an *in vitro* model.

These experiments have revealed a new avenue for research in the field of cranberry polyphenols. However, they have raised as many questions as they may have answered. More study should be conducted to elucidate the manner in which these polyphenols affect the TLR4 pathway. Also, what other inflammatory genes do

these fractions turn on? It is possible that they encourage the transcription and translation of anti-inflammatory genes as well. While in vitro experiments are a helpful way to start off in a new area of research, animal and human studies allow for stronger data. The immune modulating effects of cranberry polyphenols should be tested in an animal model to verify their validity.

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BIOGRAPHICAL SKETCH

Catherine Elizabeth Muller was born in Melbourne, Florida. Catherine grew up in Brevard County Florida and attended West Shore Junior Senior High School in Melbourne, Florida. She pursued a Bachelor of Science in food science and human nutrition with an emphasis on nutritional sciences at the University of Florida after high school and received her degree in 2007. In the spring of 2008, Catherine enrolled as a master's student in the Food Science and Human Nutrition Department at UF. While working toward her degree, she studied under Dr. Susan S. Percival. In her free time, Catherine enjoys running and the art of stained glass. After graduation, she intends on pursuing a doctor of philosophy degree in food science.