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EFFECTS OF BLACKBERRY AND BLUEBERRY POLYPHENOL EXTRACTS ON NITRIC OXIDE AND TUMOR NECROSIS FACTOR-ALPHA PRODUCTION IN LIPOPOLYSACCHARIDE-STIMULATED RAW264.7 MACROPHAGES

By

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To my little princess Sophia Ella Feresin.

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ABSTRACT

Plants are rich sources of polyphenols which are reported to play an important role in promotion of human health. Epidemiological studies have shown that populations who consume foods rich in polyphenols have lower incidence of chronic inflammatory diseases. For instance, polyphenols have been shown to modulate the inflammatory response by interacting with numerous molecular targets such as inhibiting nitric oxide (NO), and tumor necrosis factor-α (TNF-α). The objective of the present study was to investigate whether blackberry and blueberry polyphenols modulate the production of NO, TNF-α in lipopolysscharide (LPS)-stimulated RAW 264.7 macrophage cells. Berry polyphenols were extracted using ethanol and total phenolics were quantified according to the Folin-Ciocalteu method. Macrophages were treated with different doses of berry polyphenols (10, 100, 1000µg/ml) 1 hr prior to stimulation with 10ng/ml LPS for 6 hrs. Supernatants were then collected to measure NO and TNF-α production. Blackberry and blueberry extracts were confirmed to be rich in polyphenol (2000 and 3000mg/g expressed as gallic acid equivalents, respectively). Blackberry polyphenol-rich extract strongly inhibited NO production at doses of 10 and 100µg/ml by 56 and 59 %, respectively, without exerting cytotoxicity while blueberry polyphenol-rich extract had little effect on NO production. No significant decrease in TNF-α production was seen when LPS-stimulated RAW 264.7 macrophages were treated with different doses of berry extracts. These results of the present study indicate that polyphenol-rich extracts from blackberry and blueberry possess anti-inflammatory properties. In conclusion, our findings may suggest that regular consumption of polyphenol-rich berries promote human health.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Inflammation is the body's defense mechanism against infections and tissue injuries. Characterized by swelling, heat, redness, and pain, inflammation has been divided in three phases: acute, chronic, and resolution (1). Acute inflammation must be completely resolved in order to prevent it from becoming chronic (2). Chronic inflammation has been found to be the cause of diseases such as atherosclerosis, inflammatory bowel disease (IBD), cancer, rheumatoid arthritis (RA) and many others (3-6).

Steroidal anti-inflammatory drugs (SAIDS) such as glucocorticoids and non steroidal anti-inflammatory drugs (NSAIDS) such as aspirin have been extensively used to treat inflammation; however, some of these medications have been reported to have side effects. Therefore, there is a need to find alternative therapies to prevent and treat some of these inflammatory diseases. (7, 8)

In the past two decades, the focus of nutrition research has been placed on the prevention of chronic inflammatory diseases. Plants are rich sources of polyphenols which are reported to play an important role in promotion of human health (9-11). The interest in polyphenols present in foods has increased because they have been described to have numerous biological properties such as antioxidant, antimicrobial, anti-inflammatory and vasodilatory (12, 13). Also, epidemiological and experimental studies have demonstrated that populations that have diets rich in polyphenols have lower incidence of stroke, cancer, heart disease, and chronic inflammatory diseases (14-16).

Polyphenols have been shown to modulate the inflammatory response by interacting with numerous molecular targets such as inhibiting enzymes involved in: the arachdonic acid (AA) pathway, such as secretory phospholipase (sPL)-A_2, cyclooxygenase (COX)-2, lipooxygenase (LOX); and the inducible nitric oxid synthase (iNOS); and promoting a balance between pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-8, and anti-inflammatory cytokines, such as IL-10, IL-4 and transforming growth factor β (TGF-β) (5, 7, 17, 18).

Currently, studies have shown that numerous berries, e.g. blackberry, blueberry, strawberry, phenolic compounds inhibited human low-density lipoprotein (LDL) and liposome oxidation; therefore, decreasing the risk of cardiovascular disease (19). Additionally, berries have been found to have high scavenging abilities towards reactive oxygen species (ROS) and modulate the immune response in macrophage cells (20, 21). Among all fresh fruits, blueberries have one of the highest antioxidant activities due in part to its content of phenolic acids and anthocyanins (19-21). Furthermore, blueberry and blackberry phenolics have been found to inhibit NO and TNF-α production in lipopolyssacharide (LPS)-activated RAW 264.7 macrophages (22, 23). Therefore, there is a need to quantify blueberry and blackberry phenolic compounds. Furthermore, in view of the growing interest in the potential nutraceutical properties of polyphenols it seems quite relevant to investigate the anti-inflammatory properties of Highbush blueberry (*Vaccinium corymbosum L.*) and blackberry (*Rubus Spp.*) polyphenols.

1.2 Hypothesis

Berry polyphenols inhibit the production of inflammatory markers such as NO, and TNF-α, in LPS-stimulated RAW 264.7 macrophage cells. In order to test this hypothesis, we propose two specific aims:

1.3 Specific Aim 1

To determine the total polyphenol content (TPC) of fresh blueberry and blackberry. In order to achieve this aim, polyphenol extractions of fresh blueberry, and fresh blackberry were performed using ethanol extraction technique. Then, the TPH content of crude extracts was determined using the Folin-Ciocalteu method.

1.4 Specific Aim 2

To investigate the extent to which berry polyphenols suppress the production of pro-inflammatory molecules in LPS-stimulated RAW 264.7 macrophage cells. In order to achieve this aim, crude extracts of berries were tested for *in vitro* production of inflammatory biomarkers such as NO using Griess Reagent System, and TNF-α by an Enzyme-Linked Immunosorbent Assay (ELISA).

CHAPTER 2

REVIEW OF LITERATURE

2.1 Inflammation

As described in the classic literature, acute inflammation is the immunological defense mechanism triggered by infection or injuries caused by microorganisms. Following host invasion by microbes, exogenous and endogenous chemical mediators are released which brings about the basic characteristics of inflammation: rubor (redness), calor (heat), tumor (swelling), and dolor (pain). Inflammation is a reaction that occurs in the microcirculation and consists of an increase in diameter of the blood vessels to allow serum proteins and leukocytes (neutrophils, eosinophils, and macrophages) from the blood to enter the extra-vascular tissue to phagocyte microorganisms and cellular debris. This first phase of the inflammatory response is self-limited and intended to protect the host against extensive damage. However, sometimes, leukocytes are unable to fully phagocyte microorganisms and debris leading to tissue damage; thus, prolonging the acute inflammatory response. (2, 5, 7, 17)

Leukocytes, recruited to the site of the wound by chemoattractants, exogenous mediators, which includes microbial peptides and endotoxins, release the endogenous chemical mediators including eicosanoids such as prostaglandin (PG)-E_2 and leukotriene (LT)-B_4; cytokines such as TNF-α, IL-1β, IL-6, IL-10; chemokines such as IL-8, monocyte-chemoattractant protein-1 (MCP-1), and macrophage inflammatory molecule 1α (MIP1α); vasoactive amines such as histamine; adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). This pathway can be exacerbated by obvious and extreme recruitment of leukocytes to the site of the wound prolonging the inflammatory response. Once all the microorganisms and cellular debris are removed through phagocytosis, leukocytes must also be removed from inflamed sites, enabling homeostasis (resolution phase). However, in case this mechanism is not appropriately regulated, a chronic state of inflammation will persist. (2, 5, 17)

2.2 Biomarkers of Inflammation

2.2.1 Nitric Oxide

NO is a diatomic free radical that circulates freely in the membrane of cell functioning as a signaling and effector molecule. NO is synthesized from L-arginine by enzymes of the NOS family with the formation of L-citrulline. Three different isoforms have been identified so far:

endothelial nitric oxide synthase (eNOS), neural nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) with both former being constitutive. eNOS is found in the endothelium being responsible for the vasodilatory response (maintenance of low vascular tone and preveting the adhesion of platelets to the arteries walls). nNOS is found in neural tissues functioning as a neuromodulator and neuromediator. Both eNOS and nNOS are calcium dependent and essential to maintain body homeostasis. iNOS expression is stimulated by either cytokines such as IL-1β, TNF-α and γ-IFN or LPS in macrophages, neutrophils, hepatocytes, smooth muscle cells, and chondrocytes. iNOS is a calcium independent enzyme implicated in host defense due to its ability to modulate the inflammatory response. Different from eNOS, and nNOS, iNOS is capable to continuously produce a significant and toxic amount of NO. When iNOS is overexpressed, NO production is increased surpassing the physiological amounts produced by eNOS and nNOS promoting tissue injury (17, 22, 24-27). At the same time, superoxide anion (O_2^-) is also produced which reacts with NO forming peroxynitrite ($ONOO^-$) which might result in DNA damage, LDL oxidation, isoprostane formation, and etc. This indicates a possible role of superoxide dismutase as a therapeutic drug to regulate NO production during inflammation. (27)

2.2.2 Tumor Necrosis factor-α

Numerous diseases have been linked to an imbalance between the actions of pro- and anti-inflammatory cytokines that occurs due to an augment in secretion of pro-inflammatory cytokines and/or improper inhibition of cytokine effects. TNF-α is a pro-inflammatory cytokine synthesized mainly by macrophages, but it can also be expressed by other types of cells, including neutrophils, natural killer (NK) cells, T cells, and synovial cells. TNF-α is a regulatory cytokine that exerts a myriad of biological effects that lead to inflammation including activation of leukocytes, stimulation of the respiratory burst, and NO production. Furthermore, TNF-α can induce the production of chemokines and enhance the expression of adhesion molecules on the outer membrane of leukocytes and vascular endothelial cells. All these biological effects have been linked to oxidative stress, stroke and chronic inflammatory diseases such as cancer, RA and IBD. Additionally, large amounts of TNF-α have been shown to be released along with IL-1β during macrophage activation with LPS. (3, 4, 18, 23)

2.3 Inflammation – The Cause of Several Chronic Diseases

As mentioned previously, if the body is not successful in resolving the acute inflammation, a chronic state of inflammation will persist. This detrimental phase is characterized by an increased inflammatory response as the body attempts to restore its homeostasis. This increased inflammatory response can lead to further tissue and cell damage and contribute to the pathogenesis of several chronic diseases such as atherosclerosis, cancer, IBD, and RA.

2.3.1 Atherosclerosis

According to the Center for Disease Control and Prevention (CDC), diseases of the heart, which include atherosclerosis, are the leading cause of death in the U.S. (28). The American Heart Association (AHA) reports that the total cost of treating cardiovascular diseases is $273 billion and its predicted to jump to $818 billion in 20 years if prevention strategies are not implemented (29).

Atherosclerosis is a progressive disease characterized by the deposition of lipids and proliferation of smooth muscle cells in the arteries walls. High LDL-cholesterol levels, low high density lipoprotein (HDL)-cholesterol levels, cigarette smoking, oxidative stress, and genetic predisposition are the well-know causative risk factors of atherosclerosis; however, research has demonstrated that inflammation is the link between hypercholesterolemia and atherosclerosis. The formation of plaques is initiated with the recruitment of white blood cells that adheres to the intima (innermost layer of arteries). While MCP-1 has been reported to be the main contributor to leukocyte recruitment, VCAM-1 has been reported as the molecule that facilitates the adhesion of these leukocytes to the arteries walls. Also, endothelial cells selectively generate VCAM-1, in sites prompt to lesion formation, in response to an increase in blood lipids. (3, 30-32)

The transcription of the VCAM-1 gene has been reported to occur through the nuclear factor-κB (NF-κB) pathway. In fact, cytokines such as TNF-α and IL-1β have been shown to stimulate endothelial cells to generate adhesion molecules, through the NF-κB pathway, pro-inflammatory cytokines and chemoattractants (33). Additionally, TNF-α has been shown to induce oxidative stress which can result in endothelial dysfunction due to impairment of NO production (34). Conversely, superoxide dismutase has been shown to prevent oxidative stress;

thus, reducing the expression of VCAM-1 (35). Similarly, the generation of NO by eNOS has been reported to reduce the expression of VCAM-1 through the NF-κB pathway (34).

2.3.2 Cancer

Malignant neoplasms have been reported to be the second leading cause of death in the U.S. (28). According, to the National Institute of Health (NIH), the health care cost of treating cancer was $209 billion in 2005 (36). Cancer is a disease characterized by three different progressive stages; initiation, promotion and progression. Initiation is the first stage in which DNA of certain body cells undergo mutation. The promotion phase is marked by reproduction of initiated cells while the progression phase is characterized by uncontrolled cell growth (hallmark of cancer) resulting in malignant tumors. At this point, the malignant cells do not respond to normal growth regulation and metastasize to other tissues modifying their functions. (3)

Shacter *et al.* 2002 (3), has suggested that chronic inflammation may be the cause of many types of cancer. Therefore, the risk for developing cancer is increased with prolonged inflammatory state. Leukocytes are thought to produce the inflammatory mediators (metabolites of AA, cytokines, chemokines, and free radicals) responsible for inducing cell proliferation, mutation, activation, and angiogenesis.

Free radicals such as ROS have been found to have its expression increased when leukocytes are exposed to inflammatory stimuli and induce DNA damage. TNF-α has been also known to increase the formation of ROS by leukocytes (37). Additionally, lipid and protein oxidation can result in mutagenesis by promoting alterations in DNA polymerase and inhibiting enzymes involved in DNA repair (38, 39). Also, TNF-α induces the expression of iNOS and NO which can react with superoxide generating peroxynitrite. Peroxynitrite can cause DNA damage (alteration of DNA base, strand breaks, and mutation), impede DNA repair which results in leukocyte activation (40). Furthermore, the synthesis of NO produces nitrosamines which have been considered a crucial mediator of carcinogenesis stimulated during the state of chronic inflammation. Nitrosamines have been found to be extremely mutagenic and carcinogenic (41).

2.3.3 Inflammatory Bowel Disease

IBD is a disease that affects 1.4 million people in the U.S. (42). According to the CDC, this disease has an impact of $1.7 billion per year in the U.S. economy (42). IBD is a term used to describe chronic inflammation in the gastrointestinal tract. The two most common IBDs are Crohn's disease and Ulcerative Colitis. The former being characterized by inflammation

affecting the small end of the intestines while the latter is characterized by inflammation of the large intestines (43). The etiology of IBD is unknown, but the onset of this disease has been shown to occur due to an interaction between CD4+ T cells and leukocytes, mainly in response to a bacteria or food that the body does not recognize. Therefore, it starts to attack its own tissues. This will result in injury of the intestinal epithelial cells and ulceration of the intestinal mucosa. (4)

Abdominal cramping, diarrhea, nausea, fever, loss of appetite, and weight loss are some of the symptoms of IBD. Also, Crohn's disease patients might experience a greater swelling and formation of scar tissue in their intestines which will block their intestines causing cramping, vomiting, and bloating. (43)

TNF-α is a pro-inflammatory cytokine that has been implicated in the pathogenesis of IBD. Research has shown that TNF-α acts directly on the epithelial cells of the intestines by increasing the permeability of these cells, the secretion of hydrochloric acid, and the production of proteolytic enzymes by inflammatory cells which will lead to injury of epithelial cells. (4)

2.3.4 Rheumatoid Arthritis

RA is a disease that affects 1.5 million Americans (44). As reported in the Medical Expenditure Panel Survey, arthritis and its related conditions have cost the U.S. economy $128 billion in 2003, $41.8 billion more than in 1997 (45). This chronic inflammatory disease is characterized by inflammation of the lining (synovial membrane) of the joints damaging bone, cartilage, and ligaments, leading to joint deformity. Pain, swelling, and stiffness are common symptoms of this disease. RA is considered an autoimmune disease where the body attacks its own tissues. (4)

According to Standiford 2000 (4), the onset of this disease occurs due to an interaction between CD4+ T cells and macrophages which results in the release of pro-inflammatory cytokines. Particularly, TNF-α has been demonstrated to be the major cytokine involved in synovial lining hyperplasia and joint deterioration in animal and human models of arthritis (46, 47).

2.4 The use of Anti-inflammatory Drugs to Treat Inflammation

Currently, SAIDs and NSAIDs are been used to treat acute inflammation; however, these drugs have not been of benefit to treat chronic inflammatory diseases such as the ones described above. Glucocorticoids and cortisol are examples of SAIDs. These steroidal hormones are

synthesized in the outer portion of the adrenal glands (cortex) and greatly affect the metabolism of macronutrients. In fact, Laue *et al.* 1988 (48), have shown that glucocorticoids control inflammation by affecting the recruitment and modifying the function of leukocytes. It also has been found to alter the function and action of cytokines, and the metabolism of phospholipids (48). Franchimont *et al.* 2002 (49), stated that glucocorticoids exert its effects by binding to heat shock protein receptors, in the cytoplasm, that translocate to the nucleus and regulate transcription. Briegel *et al.* 1994 (50), have reported that an increase in the levels of cortisol results in suppression of pro-inflammatory cytokines. Additionally, glucocorticoids and cortisol have been shown to inhibit the synthesis of iNOS, but not its activity in endothelial cells (51). In addition, the induction of iNOS by TNF-α was also inhibited by dexamethasone, a glucocorticosteroid, in mesangial cells (52).

NSAIDs are part of a different category of drugs and they have been commonly used to treat inflammatory diseases. They exert their effect by inhibiting both isoforms of COX (COX-1 and COX-2) which are the enzymes responsible for converting AA released by PLA_2 from the phospholipid membranes into PGs and thromboxanes (TXs). COX-1 is a constitutive enzyme expressed in various tissues that appears to be responsible for the production of cytoprotective PGs that maintain the integrity of the gastric mucosa, mediate normal platelet function, and regulate renal blood flow. On the contrary, COX-2 is an inducible enzyme, highly expressed in cells involved in the inflammatory response including monocytes/macrophages, mast cells, osteoblasts, synoviocytes, chondrocytes and epithelial cells, upon stimulation with pro-inflammatory cytokines (TNF-α and IL-1) and/or LPS increasing PG production (7, 53). The inhibition of COX-1 has been shown to result in gastric ulcerations and renal failure. Meanwhile, highly selective COX-2 inhibitors increases the risk for cardiovascular diseases possibly due to TX formation via the COX-1 pathway (1, 7).

Although, NSAIDs and SAIDs have long been used to treat inflammation, these drugs have not been able to treat chronic inflammation diseases such as atherosclerosis, RA, and IBD. Also, as previously mentioned, the majority of these drugs have undesirable side effects. Therefore, it is necessary to develop alternative and safe anti-inflammatory drugs. (5, 17, 54)

2.5 Polyphenols and Prevention of Chronic Inflammation

Polyphenols are compounds that have at least one aromatic ring bearing one or more hydroxyl groups. They are abundant in the plant kingdom; therefore consisting of an important

part of the human diet. Polyphenols are normally present in seeds, skin of fruits, and stem, leaves of vegetables. They are second metabolites of plants implicated in ultraviolet radiation defense and provide plants with resistance against pathogens. Furthermore, they contribute to the sensory quality (taste, color and flavor) of fruits and vegetables (9-11). In addition, they are potent antioxidants due to their scavenging abilities towards ROS and metal chelating activities (19, 20). In addition, polyphenols have been demonstrated to possess a wide array of biological properties such as antioxidant, antimicrobial, vasodilatory, and anti-inflammatory (12, 13).

Polyphenols are often classified into three different groups: phenolic acids, flavonoids and tannins (18). Phenolic acids consist of one aromatic ring bearing one or more hydroxyl group and can be further divided into two different classes: derivatives of benzoic acid and derivatives of cinnamic acid. The content of hydroxybenzoic acid in edible plants is usually little whereas hydroxycinnamic acid is commonly present in tissue of plants. The latter consisting primarily of *p*-coumaric, caffeic, ferulic and sinapic acids; however, these acids are not usually found in their free state, occurring as glucose or most frequently quinic acid esters. For instance, caffeic acid combines with quinic acid to form chlorogenic acid which is largely found in many types of fruits including blueberries, kiwis, plums, cherries, apples, and coffee. (9, 10)

Flavonoids have the common structure of diphenylpropanes (C_6-C_3-C_6). They consist of two aromatic rings attached by three carbons generally forming an oxygenated heterocycle. They are further divided into at least six different groups according to the oxidative state of the three-carbon chain, including anthocyanins, flavanols, flavonols, flavanones, flavones, isoflavones. Anthocyanins are the most important water-soluble pigments responsible for the blue, purple, and red color of flowers and fruits. The other flavonoids, also called anthoxanthins, are normally colorless, white or yellow molecules. Catechins and epicatechins are the major flavanols found in fruits such as, apricots, whereas gallocatechin, epigallocatechin, and epigallocatechin galate are found seeds of certain leguminous, grapes, and tea. Flavonols are the most abundant flavonoids in foods being represented mainly by quercetin and kaempferol. Onions, broccoli and blueberries are rich sources of flavonols. Flavanones are abundantly found in citrus fruits such as tomatoes and prunes. Flavones, the least common of flavanoids, consist mainly of glycosylated molecules of luteoin and apigenin which are found in parsley and celery. Polymethoxylated flavanes such as tangeretin, nobeletin and sinesetin are largely found in the skin of citrus fruits, being considered the most hydrophobic flavonoids. Isoflavanes are known by its structural

similarity to estrogen. Even though they are not steroids, the position of their hydroxyl groups grant a pseudohormonal property to them allowing them to bind to estrogen receptors. As a result, they are also called phytoestrogens. Leguminous plants such as soy and soy-based products are the major source of isoflavones (genistein, daidzein, and glycitein) in the human diet. Nearly all flavonoids are glycosilated with hydroxyl substituents being attached to sugar molecules such as glucose, rhamnose, galactose and arachnose. (9, 10)

Different from phenolic acids and flavonoids, tannins are highly hydroxylated molecules of intermediate to high molecular weight. They can be divided into two distinguished groups: condensed tannins and hydrolyzable tannins. Condensed tannins or proanthocyanidins are polymers of catechins and epicatechins that form insoluble complexes with proteins which are responsible for the astringency of some foods and beverages such as grapes, apples, berries, peaches, chocolate, wine, tea, beer. Hydrolyzable tannins are polymers of gallic or ellagic acids and can be easily hydrolyzed by acid, alkali or enzymes yielding polyhydric alcohol and phenylcarboxylic acid. (9, 10)

Numerous reports published recently have emphasized the beneficial role of polyphenols as a therapeutic tool to treat acute and chronic inflammation diseases. Moreover, Cragg *et al.* 1997 (55), have reported that from 1983 to 1994, 12 out of 40 anti-inflammatory drugs approved were derivative of such natural components.

Polyphenols are abundantly present in the human diet; however, there is no accurate report on its dietary intake. In 1976, Kuhnau *et al.* (56) estimated the average dietary flavonoid intake in the United States to be approximately 1 g/day. Knekt *et al.* (14) have calculated the total intake of flavonoids to be approximately 24g/day in Finland. Similarly, Keli *et al.* (15) have found the dietary flavonoid intake to be approximately 23g/day in the Netherlands. These latter epidemiological studies have suggested that high flavonoid intake may lower the risk of chronic inflammatory diseases, and stroke, respectively.

As mentioned previously, they have been shown to modulate the inflammatory response by interacting with numerous molecular targets. Some of the possible molecular mechanisms by which polyphenols exert their anti-inflammatory activities have been suggested to include: the inhibition of eicosanoids producing enzymes such as PLA_2, COX and LOX which will result in reduced levels of PGs and LTs; the inhibition of the NOS family; and a balance between pro-

inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) and anti-inflammatory cytokines (IL-10, IL-4, TGF-β). (5, 7, 17, 18, 54)

2.6 Blueberry and Blackberry Composition and Their Anti-inflammatory Properties

Blueberries and blackberries are both native to North America and known for similar health benefits. Blueberries belong to the *Ericaceae* family whereas blackberries belong to the *Rosaceae* family (57). Both fruits have shown to possess anti-inflammatory properties which is suggested to be due their high polyphenolic content (57-60).

2.6.1 Blueberry

Blueberries, specifically, *Vaccinium* berries, have been reported to have a high content of flavonoids including anthocyanins, which occur as glycosides, and flavonols (quercetin, kaempferol, and myercitin). The former has been demonstrated to be the main functional constituent of blueberries. This fruit has also a great content of phenolic acids (chlorogenic, ferulic, and caffeic acids). In addition, among many fruits and vegetables, blueberry has exhibited one of the greatest antioxidant activities in vitro. (57-59) Furthermore, Fukomoto & Mazza 2000 (60), have shown that the antioxidant capacity of polyphenols increases with an increase in hydroxyl groups.

Wang & Mazza 2002 (22), have reported that polyphenols extracted from blueberries such as myercitin and quercetin decreased NO production in LPS-stimulated RAW264.7 macrophage cells. Also, according to Wang & Mazza 2002 (23), blueberry anthocyanin-rich extracts induced the production of TNF-α and modulated the inflammatory response in RAW264.7 LPS/γ-IFN-activated macrophages.

Similarly, blueberry extract inhibited NO production in LPS-activated BV2 microglia with IC_{50} value of 100µg/ml. iNOS protein levels were also inhibited by phenolics in the blueberry extract. In the same study, when compared to control blueberry extract was also shown to decrease TNF-α levels at a dose of 500µg/ml. (61)

2.6.2 Blackberry

Similar to blueberries, blackberries have been reported to have great antioxidant capacity. They are rich in anthocyanins (with the major compounds being cyanidin-based), phenolic acids (ellagic acid and its derivatives) and hydrolyzable tannins (ellaginotannins, and gallotannins). (62)

Wang & Mazza 2002 (22), have shown that crude extracts from blackberries were able to moderately suppress NO production in LPS-stimulated RAW 264.7 macrophages. However, the same crude extract had little effect on TNF-α production. The concentrates exhibited significant effect on TNF-α production when compared to crude extracts; yet TNF-α induction of berry anthocyanin-rich extracts in stimulated macrophages was observed at higher doses (250 and 500 μg/ml). (23)

Furthermore, Pergola *et al.* 2006 (63), have shown that NO production decreases significantly in J774 macrophage cells stimulated with LPS after the addition of blackberry anthocyanin-rich extract. Similarly, Cuevaz-Rodriguez *et al.* 2010 (64), have reported that blackberries anthocyanin-rich extract significantly inhibited NO production in LPS-stimulated macrophages without exerting any cytotoxic effect.

CHAPTER 3

MATERIAL AND METHODS

3.1. Extraction of Polyphenols from Fresh Berries

Polyphenols were extracted according to the method described by Kim & Lee 2002 (65). Briefly, 50g of fresh berry were combined with 1g of ascorbic acid and 100ml of absolute ethanol and macerated for 3 min at high speed in a blender. The mixture was transferred to a 500-ml beaker and combined with 50ml of 80% ethanol. Then, the beaker was placed in an ice bath and the mixture was homogenized using a Polytron set at 7 for 2 min. The mixture was filtered through a Whatman #2 filter paper using a chilled Bϋchner funnel under vaccum suction. The residue was transferred into a 500-ml beaker (placed in an ice bath) with 100ml ethanol and the mixture was again homogenized using the Polytron. The mixture was once again filtered through a Whatman #2 filter paper using a chilled Bϋchner funnel under vaccum suction. Then, the two filtrates were combined and transferred into a 1-L round-bottom flask with an additional 50ml of 80% ethanol. The solvent was evaporated using a rotary evaporator (Rotavapor R-3000, Buchi, Switzerland) at 62ºC and 50rpm, until the volume in the flask reached 10-30ml. The extract was stored in -80ºC for 24 h and subsequently freeze-dried (Virtis Benchtop K Freeze dryer, Stone Ridge, NY). The crude extracts were stored in -20ºC for further analysis.

3.2. Determination of Total Polyphenols

The phenolic concentration was measured using the Folin & Ciocalteu method previously modified by Kim *et al.* 2002 (66). In brief, 1ml of properly diluted extracts and standard solutions of gallic acid (0, 50, 100, 150, 200, 250mg/L) were added to a 25ml volumetric flask containing 9 ml of deionized water (ddH_2O). One milliliter of Folin & Ciocalteu's phenol reagent were added to the mixture and shaken using a vortex. After 5-min incubation, 10ml of 7% Na_2CO_3 solution was added and mixed using a vortex. The solution was immediately diluted to 25ml using ddH_2O and mixed thoroughly. A reagent blank was prepared using 10ml of ddH_2O. Following incubation for 90 min at room temperature, the absorbance versus prepared blank was read at 750nm using a spectrophotometer (Ultrospec™ 2100 *pro* UV/Visible Spectrophotometer, GE HealthCare Lifesciences, UK). The TPH content of freeze-dried berry's extracts was expressed as mg of gallic acid equivalents (GAE)/g of powder. All samples were analyzed in triplicate.

3.3. Cell Culture

The RAW 264.7 mouse macrophage cell line [American Type Culture Collection (ATCC), Manassas, VA] was cultured in Dulbelcco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and 1% antibiotics (streptomycin and penicillin). All cultures were incubated at 37 ºC in a humidified atmosphere with 5% CO_2 and 95% air. Cell number was assessed by tripan blue dye exclusion on a Levy hematocytometer (Hau, PA). Cells were grown to 80% confluence in sterile cell culture flasks and gently detached using a scraper (BD Falcon, Bedford, MA). For phenolic compounds treatment tests, cells were cultured in triplicate in Cellstar flat-bottom cell culture plates (Greiner Bio One, Monroe, NC). Cells were plated at density of 2 x 10^4 cells/well in 24-well cell culture plates and grown for 24 h to allow them to attach to the plate. The compounds tested were originally dissolved in DMEM to make solutions in the following concentrations: 0, 10, 100, 1000 and 10,000μM. Cells were supplemented with the test compounds for 1 h before stimulation with 10ng/ml LPS. The activated cells were further incubated for 6 h. Then, supernatants were collected to determine nitrite concentration, and TNF-α activity and/or stored at –80ºC for further use. Control cells were grown under equal conditions but were not exposed to the test compounds or LPS.

3.4. Nitric Oxide Determination

Griess Reagent System (Promega, Madison, WI) was used to measure nitrite concentration as described by the manufacture. Sodium nitrite standard or 50μl of culture supernatant was combined with an equal volume of sulfanilamide solution, in triplicate, and incubated at room temperature for 5 min, protected from light, using a 96-well plate. Then, 50μl of *N*-(1-naphthyl)-ethylenediamine dihydrochloride was added to all wells. After 5-min incubation at room temperature, protected from light, the absorbance was read at 540nm using a microtitation plate reader.

3.5. TNF-α Quantification

TNF-α in cell supernatants was determined using a Quantikine mouse TNF-α ELISA kit that was carried out as specified by the manufacture (R&D Systems, Inc., Mineapolis, MN). Fifty microliters of assay diluent were added to each well and combined with an equal volume of standard, control or sample and gently mixed by tapping the plate frame for 1 min. After 2-h incubation at room temperature, wells were aspirated and washed five times using a washer buffer (400μl). Subsequently, 100μl of mouse TNF-α Conjugate were added to each well and the

plate was incubated for another 2 h at room temperature. The aspiration/wash process was repeated and 100µl of substrate solution was added to each well. Following 30-min incubation, protected from light, 100µl of Stop solution was added to each well. Absorbance was read at 540nm within 30 min of stopping reaction on a microplate reader.

3.6. Cell Viability

Cell viability was measured using the yellow tetrazolium salt (MTT) assay as described by the manufacturer (ATCC, Manassas, VA). In brief, RAW 264.7 macrophages were plated at 1 x 10^5/well in a 96-well plate and incubated overnight. Then, cells were treated with berry polyphenols in doses of 0, 10, 100, 1000 and 10,000µg/ml for 1 h before stimulation with LPS (10ng/ml). After 6 hrs of treatment, supernatant was aspirated, and 100µ1 of medium was added to each well followed by 10µl of MTT reagent. The plate was then incubated for 4 h until purple precipitated was visible. Later, 100µl of detergent reagent were added to each well and the plate was gently swirled and left with cover in the dark at room temperature overnight. The absorbance was measured at 570nm in a microplate reader.

3.7. Statistics

The data were analyzed using analyzes of variance (ANOVA) followed by Tuckey's post hoc pairwise comparisons to determine which means were significant different. SPSS version 18 (IBM, Chicago, IL) was used for all statistical analyses. Significance was accepted at $p \leq 0.05$.

CHAPTER 4

RESULTS

4.1. Total Polyphenolic Content

The TPC of freeze-dried blueberry and blackberry extracts were 55.56 and 79.06 mg of GAE/g of berry powder, respectively.

4.2. Nitric Oxide Production

LPS significantly increased NO levels in macrophages when compared to control (P = 0.021). When compared to LPS blackberry extracts at doses of 10 and 100µg/ml strongly inhibited NO production by 56 (P = 0.042) and 59% (P = 0.029), respectively (Figure 1).

LPS did not significantly increase NO levels in macrophages when compared to control (P = 0.224). When compared to LPS, blueberry extracts decreased NO production at doses of 10 and 100µg/ml by 47 (P = 0.577) and 67% (P = 0.234), respectively; albeit, the inhibition was not statistically significant (Figure 2).

4.3. Tumor Necrosis Factor-α Production

LPS significantly increased TNF-α levels in macrophages treated with blackberry and blueberry polyphenols when compared to control (P = 0.000) (Figure 3 and 4). When compared to LPS blackberry extracts at doses of 10, 100 and 1000µg/ml were able to decrease TNF-α production by 11 (P = 0.431), 14 (P = 0.249), and 16% (P = 0.136), respectively; albeit, the decreases were not statistically significant (Figure 3). In addition, when compared to LPS, blueberry extracts decreased TNF-α production at dose of 100µg/ml by 14% (P = 0.853); however, the decrease was not statistically significant (Figure 4).

4.5. Cell Viability:

Neither LPS (10ng/ml) nor berry extracts in doses of 10, 100, and 1000µg/ml did decrease cell viability (Figure 1, 2, 3, and 4).

CHAPTER 5

DISCUSSION

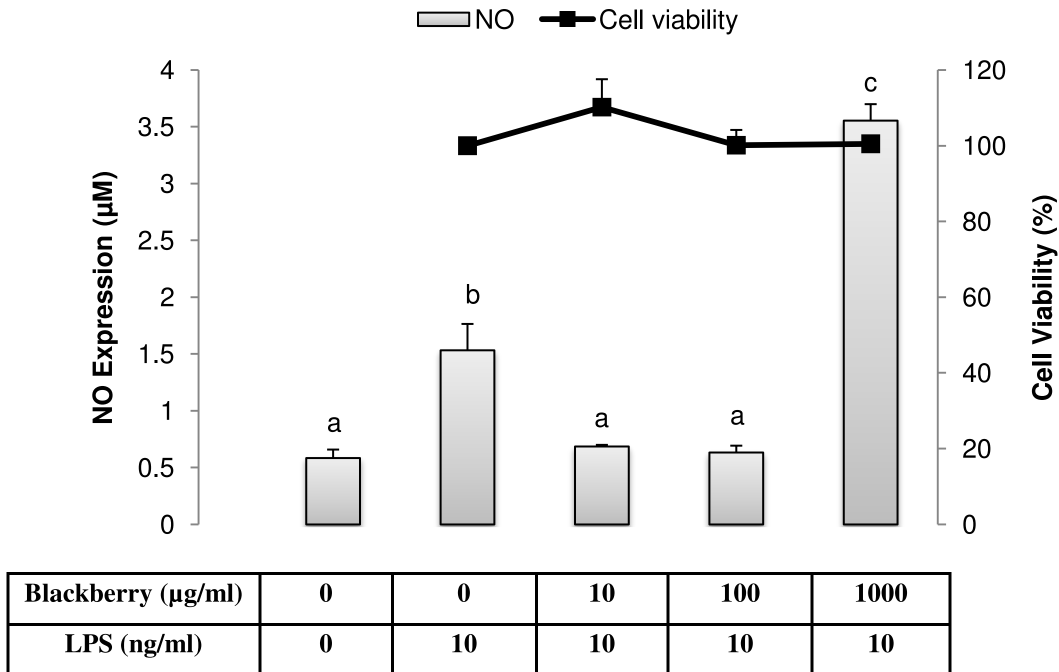
This study examined the anti-inflammatory properties of both blackberry and blueberry-rich polyphenol extracts using RAW264.7 macrophages. The findings of the present study indicate that blackberry extract significantly inhibit the production of NO without having any cytotoxic effect at doses ranging from 10 and 100µg/ml. These results support the study of Pergola *et al.* 2006 (63) where it was demonstrated that blackberry extract, which was found to be rich in cyanidin-3-*O*-glucoside (C3G), was able to suppress NO production in J774 cells stimulated with LPS. They also have demonstrated that blackberry extract inhibited iNOS expression and activity in rat lungs stimulated with LPS. Similarly, Cuevaz-Rodriguez *et al.* 2010 (64), have reported that blackberry polyphenols inhibit NO production in RAW264.7 macrophages stimulated with LPS without any cytotoxic effect up to 50µM. Pergola *et al.* 2006 (63) concluded that the decrease in NO production was due to inhibition of iNOS expression, which is regulated at the transcriptional level mainly through reduction in the activation of the NF-κB pathway. Hori *et al.* 2001 (67), have stated that the decrease in NO production by blackberry extract was a result of an attenuation in ROS production, likely due to C3G scavenging property. Likewise, Serraino *et al.* 2003 (68), have reported that C3G blackberry rich extract inhibited the formation of peroxynitrate (free radical) as demonstrated by a decrease in mitochondrial respiration and DNA damage preventing endothelial dysfunction.

Our study has also shown that blueberry extract decreases the production of NO in LPS-stimulated RAW264.7 macrophages without having any cytotoxic effect ranging from doses of 10 and 100µg/ml; however, these decreases were not statistically significant. Also, Wang & Mazza 2002 (22), have reported that blueberry extract exerts no effect on NO production in LPS-stimulated Raw264.7 macrophage cells. However, they reported that when blackberry extract were further purified they exert greater inhibitory activity on NO production than blueberry extract. In addition, this outcome is attributed to the differences in the phenolic profile of each berry (22). For example, while blackberry extract contains higher amounts of glycosylated cyanidin, blueberry extract are rich in glycosydes of cyanidin, malvidin, malvidin-3-glucosyde, and delphinidin.

Furthermore, the present study may suggest that blackberry extract at doses of 10 and 100µg/ml, as well as blueberry extract at of dose of 100µg/ml, reduces TNF-α production in LPS-stimulated Raw264.7 macrophages when compared to untreated cells; albeit, the decrease was not statistically significant. In contrast, Wang & Mazza 2002 (23), have reported that blackberry extract has no effect on the production of TNF-α in the same type of cells, which was probably due to their low concentration of polyphenols. However, in the same study when the extracts were further purified (the concentration of polyphenols was increased), TNF-α production increased. Based on these results, they have concluded that the regulation of NO and TNF-α production in RAW264.7 macrophage cells is regulated through different pathways. However, based on our data we speculate that the activation of TNF-α does regulate NO production; however, its regulation may occur via other pathways. This argument agrees with Pergola *et al.* 2006 (63) who reported that TNF-α increases NO production by inducing iNOS expression and/or activity (63).

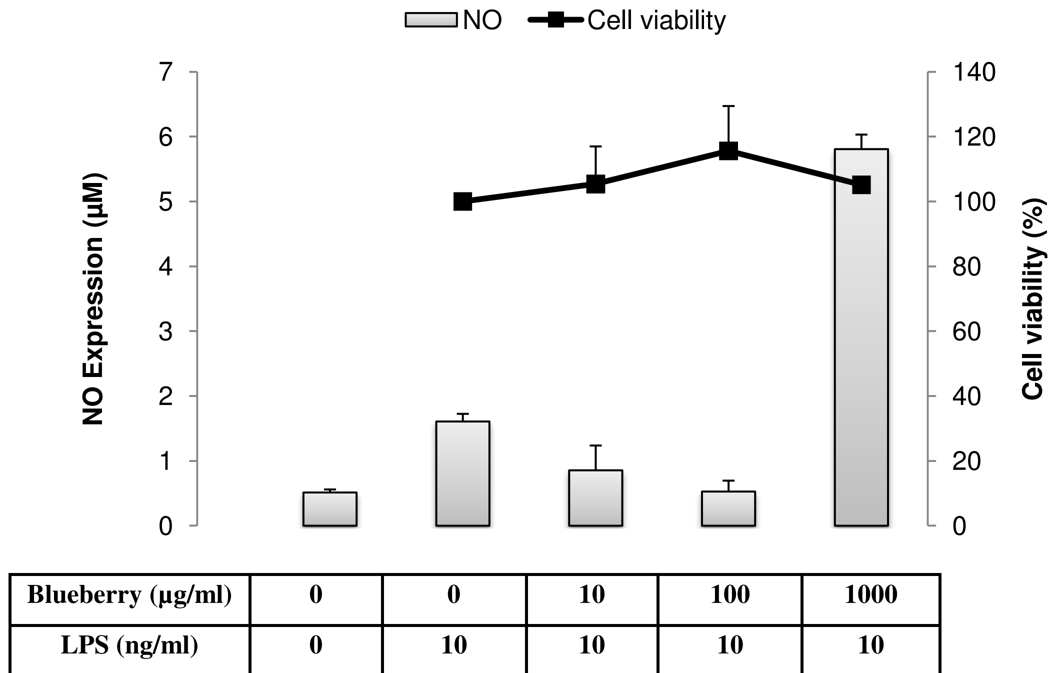
In conclusion, the results of the present study indicate that blackberry and blueberry polyphenol-rich extracts possess anti-inflammatory properties suggesting that regular consumption of polyphenol-rich berries promotes human health. In addition, the results implicate these extracts in the development of new anti-inflammatory drugs. Further research is needed in order to elucidate the exact mechanisms by which polyphenols exert its anti-inflammatory effects.

FIGURES



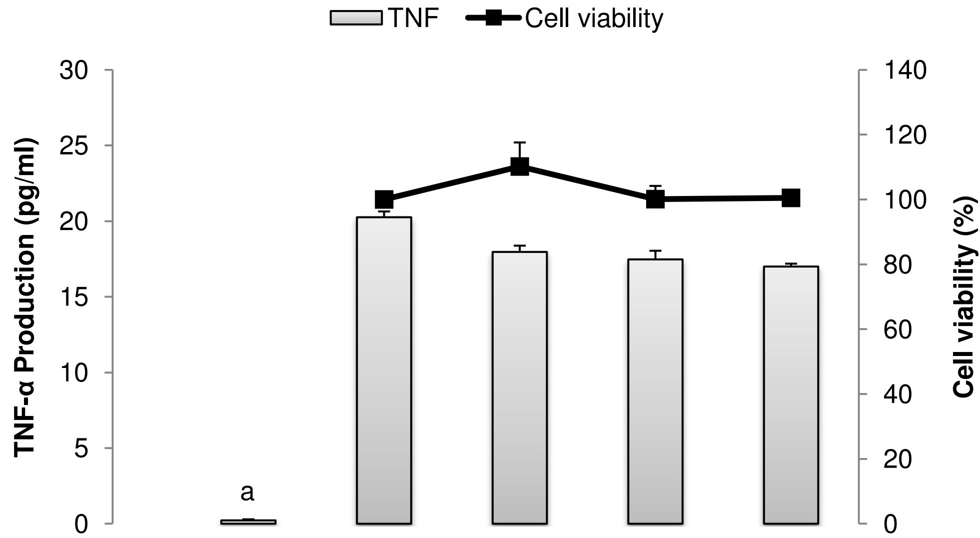
Blackberry (µg/ml)	0	0	10	100	1000
LPS (ng/ml)	0	10	10	10	10

Figure 1. Effect of blackberry extract on NO production in LPS-stimulated RAW 264.7 macrophages. Cell viability of LPS was arbitrarily set as 100, and all the others were normalized to this LPS control value. Bars represent the mean of triplicate ± se. Bars with different letters denote significant difference in NO levels ($P < 0.05$).



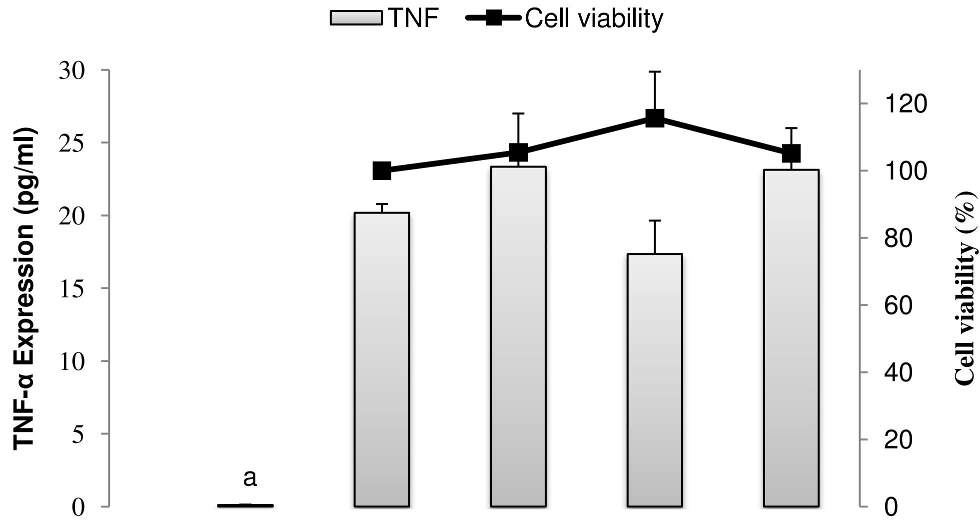
Blueberry (µg/ml)	0	0	10	100	1000
LPS (ng/ml)	0	10	10	10	10

Figure 2. Effect of blueberry extract on NO production in LPS-stimulated RAW 264.7 macrophages. Cell viability of LPS was arbitrarily set as 100, and all the others were normalized to this LPS control value. Bars represent the mean of triplicate ± se. Bars with different letters denote significant difference in NO levels ($P < 0.05$).



Blackberry (μg/ml)	0	0	10	100	1000
LPS (ng/ml)	0	10	10	10	10

Figure 3. Effect of blackberry extract on TNF-α production in LPS-stimulated RAW 264.7 macrophages. Cell viability of LPS was arbitrarily set as 100, and all the others were normalized to this LPS control value. Bars represent the mean of triplicate ± se. Bars with different letters denote significant difference in TNF-α levels ($P < 0.05$).



Blueberry (μg/ml)	0	0	10	100	1000
LPS (ng/ml)	0	10	10	10	10

Figure 4. Effect of blueberry extract on TNF-α production in LPS-stimulated RAW 264.7 macrophages. Cell viability of LPS was arbitrarily set as 100, and all the others were normalized to this LPS control value. Bars represent the mean of triplicate ± se. Bars with different letters denote significant difference in TNF-α levels ($P < 0.05$).

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BIOGRAPHICAL SKETCH

Rafaela G. Feresin

Rafaela G. Feresin was born on August 24, 1981 in Goiânia, Goiás, Brazil. She is the daughter of Jose H. Feresin and Patricia F. Gonçalves and the older sister of Marcela G. Feresin and Jordana G. Feresin. She is also the mother of beautiful little girl, Sophia Ella Feresin. She graduated from high school in 1998 where she attended in Goiânia, Goiás, Brazil. She received her bachelor's degree in Nutrition from The Universidade Federal de Goiás, Brazil in March of 2004. Then, she joined The Florida State University during the fall semester of 2009 when she started her graduate studies under Dr. Bahram H. Arjmandi's mentorship. While doing her master's she was a graduate assistant and her work focused on the anti-inflammatory properties of blueberry and blackberry polyphenols using LPS-stimulated RAW264.7 macrophage cells. During this time she was also a teaching assistant as she taught the course HUN1201 (Science of Nutrition).