ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS OF SELECTED FRUITS IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

A Thesis

by

EMILY DURHAM TOWNSLEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	Susanne Mertens-Talcott
Committee Members,	Stephen T. Talcott
	David H. Byrne
Interdisciplinary Faculty Chair,	Alejandro Castillo

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ABSTRACT

Anti-inflammatory and Antioxidant Effects of Selected Fruits in Human Umbilical Vein Endothelial Cells. (December 2010)

Emily Durham Townsley, B.A., Texas A&M University

Chair of Advisory Committee: Dr. Susanne Mertens-Talcott

Cardiovascular Disease (CVD) is of great concern to the American population as it is the leading cause of death in the United States. Vascular inflammation can lead to many outcomes, one of which is the development of atherosclerotic lesions – a common manifestation of CVD. The purpose of this study was to determine the antioxidant and anti-inflammatory effects of secondary plant compounds (SPC) extracted from fruits, specifically investigating their effects as a preventative measure in vascular lesion development. Antioxidant activity (AOX) was measured using the ORAC assay, quantifying antioxidant enzymes and determining protection against reactive oxygen species (ROS) *in-vitro.* Anti-inflammatory activity was measured through quantifying mRNA expression of pro-inflammatory markers in LPS-induced Human Umbilical Vein Endothelial cells (HUVEC).

Stone fruits contain a significant amount of SPC in their flesh and skin. This study suggested that SPC present in Black Splendor plum (BS), Crimson Lady peach and Rich Lady peach extracts exert protective effects on HUVEC. Total extracts of the fruit, as well as anthocyanin and non-anthocyanin fractions were investigated. The SPC extracts of the stone fruits were proven to have high AOX based on their ability to scavenge free radicals, indicating anthocyanin compounds eliciting the highest effect. Further investigation established significant protection against *in-vitro* ROS, indicating BS-total extract providing the most protective effect. Anti-inflammatory effects were

seen with decreased expression of LPS-induced inflammatory markers (NF-κB, IL-6, IL-8, V-CAM, COX-2) with pre-treatment of peach and plum extracts. Results are suggestive that certain SPC elicit protective effects targeting specific pro-inflammatory markers.

Superfruits, a rising research interest within the functional food industry, are known for their SPC content. AOX was seen through a suppression of the superoxide-radical generating enzyme, NADPH oxidase and reduction in antioxidant enzyme, superoxide dismutase (SOD). Pomegranate from China and Turkey, and both açai varieties elicited the largest decrease in expression of NADPH oxidase. Pomegranate grown from China, Turkey and the US, and all goji extracts elicited the highest suppression of SOD enzyme activity. The highest suppressive effect of pro-inflammatory biomarkers (NF-κB, TNFα, IL-6, and IL-8) was seen was seen with treatment of pomegranate grown in India. To the best of our knowledge, this was the first account of anti-inflammatory activity relating to preventative effects of inflammatory lesion development.

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1. INTRODUCTION

Within the past decade public interest in functional foods has grown considerably. Consumers are looking for ways to improve their overall health and well being through preventative measures. Fruits and vegetables are considered to be among these functional foods because they contain bioactive compounds that possess antioxidant capabilities. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, and have been thought to reduce the body's inflammatory response as well (*1, 2*).

Cardiovascular Disease (CVD) is of great concern to Americans as it is a leading cause of death in the United States. Metabolic Syndrome refers to a cluster of risk factors commonly associated with CVD; therefore a person diagnosed with Metabolic Syndrome is at high risk to develop CVD later in life. Metabolic Syndrome is defined by the American Heart Association and the National Heart, Lung, and Blood Institute; as having any three of these five risk factors. These risk factors include elevated waist circumference, elevated triglycerides, reduced HDL (high-density lipoprotein) cholesterol, elevated blood pressure, and elevated fasting glucose (*3*). According to data collected from 2003-2006 by the National Center for Health Statistics, 34% of adults age 20 and older met the criteria for Metabolic Syndrome. Because of the correlation in risk factors between CVD and Metabolic Syndrome, the increasing incidences of Metabolic Syndrome in the US are a preview to the potential increase in incidence of CVD to come.

Inflammatory processes may lead to many outcomes, one of which is the development of atherosclerotic lesions – a common manifestation of CVD (*3-5*). An inflammatory response can be stimulated by a series of biochemical events such as oxidative stress, vascular stress, or physical injury to the vasculature. There are two categories of inflammation, acute and chronic. Acute inflammation is a controlled

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This thesis follows the style of *Journal of Agriculture and Food Chemistry*.

response to pathogens for a short period of time while chronic inflammation occurs over an extended period of time and can be detrimental to the body.

Vascular disorders such as these are propagated by inflammation through over-expression of adhesion molecules and cytokines. These are thought to participate in the pathogenesis of atherosclerosis, in which a buildup of fatty substances, cholesterol, cellular waste products and other substances on vasculature linings, generating a plaque. This stimulates cell signaling pathways to further perpetuate the inflamed vascular lesion. These plaques may restrict blood flow and can also burst, potentially causing a blood clot (*4, 6-8*). The primary mediators for inflammation are reactive oxygen species and cellular signaling molecules such as cytokines, transcription factors, growth factors, and cell adhesion molecules (*9*).

Endothelial cells are used for *in-vitro* assays to determine inflammatory responses (*10-12*). Endothelial cells line the vasculature of the circulatory system and are involved in the processes of inflammation, vasculogenesis, and vasoregulation. By challenging human umbilical vein endothelial cells (HUVEC) with lipopolysaccharide (LPS), pro-inflammatory receptors are stimulated leading to activation of specific transcription factors. Transcription factor activation leads to production of pro-inflammatory signaling molecules which further perpetuate the inflammatory process. HUVEC cells are pre-treated with selected fruit extracts in attempts to disrupt or decrease activity of the pro-inflammatory mechanism. The expression of inflammation biomarkers along the pro-inflammatory cell signaling pathway in HUVEC cells pre-treated with fruit extract were quantified and measured against untreated HUVEC cells. This information gives insight into the anti-inflammatory effect of the fruit extract on HUVEC cells in a pro-inflammatory state.

Stone fruits, specifically peaches and plums, contain a significant amount of bioactive compounds in their flesh and skin. Previous studies have investigated the antioxidant effects of stone fruits showing significant antioxidant activity (*13*). Further studies have also shown stone fruits to possess anti-cancer activity in breast cancer cells (*14*). The results of previous investigations have indicated a need for the bioactive

compounds in peach and plum fruit to be further investigated, analyzing the potential use as preventative or protective agents towards cardiovascular disease, specifically suppressing the CVD related inflammatory pathways.

Research investigating the health benefits of exotic fruits has grown considerably. Consumer interests are trending towards eating a healthy and well-balanced diet, creating a large market for health beneficial foods. Exotic fruits, such as açai, pomegranate and goji, are known to be dense in secondary plant compounds. There is minimal research on the anti-inflammatory effects regarding CVD for açai, pomegranate, and goji. The potential marketability in the health industry of these fruits can be great given the proper scientific basis for justification.

The purpose of this study was to determine the antioxidant and anti-inflammatory effects of bioactive compounds extracted from selected stone and exotic fruits. The aim of the investigation was to understand the secondary plant compound composition and how it affects the biological activities targeting CVD-related inflammatory pathways. The intent of the results from this study was to provide knowledge of stone fruits (peaches and plums) and exotic fruits (açai, pomegranate and goji) for potential use as preventative measures towards CVD.

Specific objectives were:

1. To determine the antioxidant and anti-inflammatory effects of stone fruits *in-vitro*, targeting Cardiovascular Disease-related pathways. The total extract was analyzed as well as the anthocyanin and non-anthocyanin polyphenolic fractions.

2. To establish the bioactivity of secondary plant compounds found in exotic fruits (açai, pomegranate, and goji), giving a more in-depth understanding of the anti-inflammatory and antioxidant mechanisms targeting Cardiovascular Disease.

2. LITERATURE REVIEW

2.1. Cross talk between inflammatory and oxidative pathways leading to vascular stress

The pathogenesis of vascular disorders leading to atherosclerosis has been heavily researched and shown to be multifaceted. The majority of the research has been focused on inflammatory pathways, however this mechanism works congruently with oxidative stress mechanisms (*2, 12*). The two mechanisms of focus for this study work cyclically towards atherosclerotic lesion development.

Inflammation is an organism's defense mechanism against harmful pathogens. Chronic inflammation occurs over an extended period of time and can lead to vascular disorders. An inflammatory response can be stimulated by a series of biochemical events such as oxidative stress, vascular stress, and inflammatory mechanism mediators. The primary mediators for inflammation are signaling molecules (cytokines, transcription factors, growth factors, and cell adhesion molecules). The activation and mediation of these signaling molecules has been proposed to be further perpetuated in an environment of oxidative stress, such as a higher level of reactive oxygen species (ROS).

2.2. Development of an atherosclerotic region

Vascular lesions have been shown to start as "fatty streaks" (*2*). Low-density lipoproteins (LDL) which are naturally present in the bloodstream shuttle in and out of the sub-endothelial space due to their amphiphilic nature. The initial stage of a vascular lesion begins in a state of oxidative stress where the LDL undergoes oxidative modification (ox-LDL) and becomes trapped in the sub-endothelial space (*2*). The presence of ox-LDL may stimulate the NF-κB mediated generation of cell adhesion molecules (CAM), as seen in Figure 2.1. The two main CAMs are intracellular cell adhesion molecules (I-CAM-1) and vascular cell adhesion molecules (V-CAM-1) which are expressed on the cellular membrane (*15*). Their role is to mediate the adhesion of monocytes to the vascular endothelium and move them to the sub-endothelial space.

Monocytes are a type of white blood cell that responds to an immune response, such as inflammation. Once the monocytes have entered the sub-endothelial space they differentiate into macrophages, accumulating the ox-LDL via endocytosis to become foam cells which aggregate causing endothelial dysfunction and tissue injury (Figure 2.1). Macrophages have a negative feedback control which regulates the uptake of LDL, however this is bypassed by ox-LDL (*2*). Macrophages generate reactive oxygen species (ROS) generating a pro-oxidant state.

This cyclic mechanism suggests that antioxidants scavengers will decrease the pro-oxidant state, suppressing the inflammatory pathway leading to pathogenesis of vascular lesions (*16*).

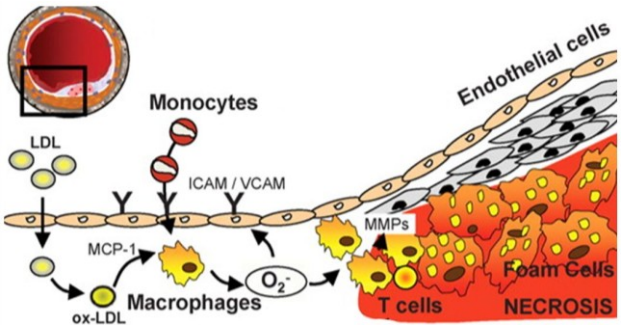


Figure 2.1 Pathogenesis of atherosclerosis in endothelial cells (*16*).

2.3. NF-κB mediated inflammatory pathway

There are many proposed mechanisms that mediate an inflammatory process. The mechanism of focus for this research responds to extracellular stimuli, Lipopolysaccharide (LPS), initiating a cell signaling pathway (CSP). This pathway leads to modulation of gene expression for pro-inflammatory cytokines and cell adhesion molecules (Figure 2.2). LPS is found in the outer membrane of gram-negative bacteria and is shown to elicit a strong immune response, activating a specific toll-like receptor, TLR-4. Through a series of CSP transducers, including interleukin-1 receptor-associated

kinase 1 (IRAK-1) and TNF receptor associated factor-6 (TRAF-6), the transcription factor NF-κB is up-regulated. Transcription factors are proteins that bind to specific DNA sequences and control the transcription of DNA to RNA, therefore determining protein production. NF-κB is located in the cytoplasm and is comprised of p50 and p65 units, as well as an inhibitor molecule. Upstream activation, in this case up-regulation of toll-like receptor-4 (TLR4) due to the LPS challenge, initiates CSP transduction involving IRAK-1 and TRAF-6, modulating the detachment of the inhibitor molecule of NF-κB. This allows for translocation of the NF-κB p50 and p65 units into the nucleus where transcription occurs. NF-κB can also be activated via cell signaling pathways beginning with oxidized lipids (ie, ox-LDL) or cytokine stimulation (Figure 2.2) (*17*). Activated NF-κB modulates the production of multiple pro-inflammatory cytokines (Interlukin-6 and Interlukin-8) and cell adhesion molecules (CAMs) (*17*). This develops into a positive feedback loop - as more cytokines are produced due to the activation of NF-κB through pro-inflammatory stimuli (LPS), NF-κB activating pathways will be stimulated in response to cytokines.

LPS is also an especially potent stimulus for Tumor necrosis factor alpha (TNFα) synthesis (*18*). TNFα is an inflammatory pathway mediating cytokine which binds TNF receptors type II, leading to activation of transcription factor NF-κB. Signals are transmitted through the recruitment of more than a dozen different signaling proteins, which together form signaling cascades (*19*).

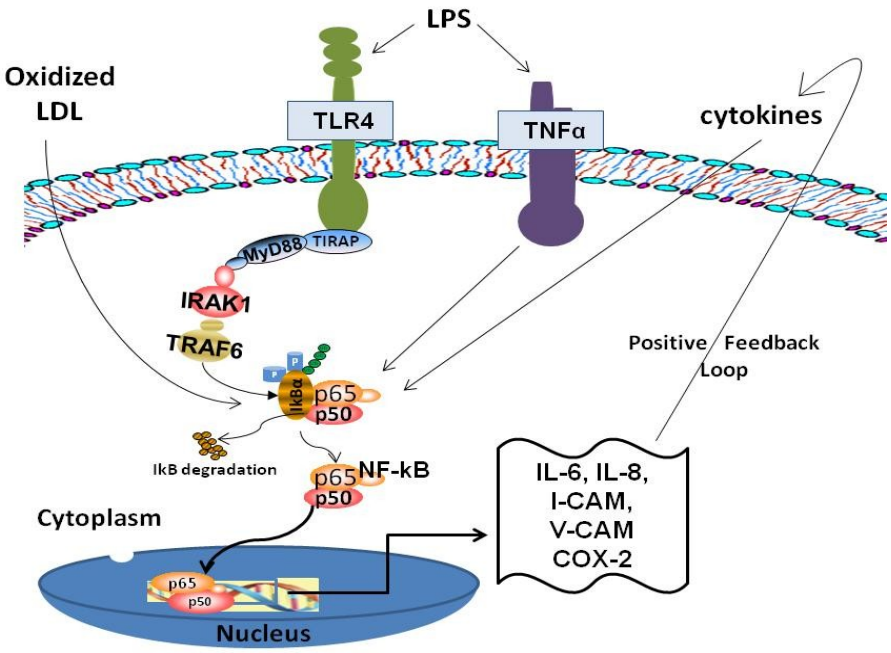


Figure 2.2 Molecular pathway in LPS activated membrane receptors TLR4 and TNFα. These receptors engage cell signaling pathways which lead to NF-κB activation. NF-κB activation mediates the production of pro-inflammatory signaling molecules (IL-6, IL-8, V-CAM, I-CAM, and COX-2). IL-6 further perpetuates NF-κB activation, creating a positive feedback loop. NF-κB is red-ox sensitive and can be activated in the presence of oxidized lipids as well.

2.4. Active roles of inflammatory biomarkers

2.4.1. Toll-like Receptor-4 pathway

Toll-like Receptor-4 (TLR-4) is located on the cell membrane and responds to an LPS challenge and is therefore important in the activation of an immune response. Once activated, this protein initiates a cell signal transduction pathway. Downstream from TLR-4 is the protein-kinase, IRAK-1 as well as the protein TRAF-6, which both mediate the signal transduction pathway leading to NF-κB activation (*20*).

2.4.2. Nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB)

NF-κB is a transcription factor that controls the transcription of DNA. Upon stress and cell damage, NF-κB is increased in response to regulating the immune

response to infection or damage. NF-κB is increased in situations of chronic inflammation, cancer, and cardiovascular disease due to activation of inflammatory mechanism mediators (*2, 8, 17*). Plant polyphenolic compounds have been demonstrated to decrease inflammation through suppression of NF-κB mediated pathways (*21*).

2.4.3. Tumor necrosis factor-alpha

TNF-α is a cytokine involved in general inflammation. It regulates the interaction of immune cells and can lead to cell death. TNF-α has been found to be continually up-regulated in situations of chronic inflammation, cancer and cardiovascular disease. Polyphenolic compounds have been demonstrated to decrease TNF-α in these pathogenic situations (*22*).

2.4.4. Interleukins 6 and 8

Interleukin-6 (IL-6) is a cytokine that acts as a pro-inflammatory signaling molecule. It is produced as a result of NF-κB activation in response to inflammation. Pro-inflammatory cytokines can stimulate production of pro-inflammatory molecules such as IL-8, VCAM-1, and ICAM-1. Interleukin-8 (IL-8) is a chemokine involved in the regulation of inflammation and cell-cell interactions. Chemokines are signaling molecules which attract an immune response to the vascular lesion.

2.4.5. Vascular and intracellular cell adhesion molecules

Intracellular cell adhesion molecule (I-CAM-1) and the vascular cell adhesion molecule (V-CAM-1) mediate the adhesion of monocytes to the vascular endothelium, followed by transmigration into the sub-endothelial space (*16*).

2.4.6. Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) is an inflammatory mediator which helps to maintain a persistent state of inflammation. This signaling molecule is mediated through NF-κB activation and is often over-expressed in inflammatory lesions (*9*).

2.5. General information of stone fruits

The following is a brief overview of the selected fruit for this study describing the horticultural background, secondary plant compound composition, and potential bioactivities – according to published literature.

Peaches and plums have been widely researched providing a plethora of information on their chemical composition. Peach and plum fruits are both classified as stone fruits in the genus, *Prunus*. Due to the similarity in composition between peach and plum fruits, they will be reviewed together.

Peaches (*Prunus persica*) were domesticated in China and mentioned in Chinese folklore as far back as 3,000 years ago. Then, beginning about 2,000 years ago the peach spread to Europe. It was later taken to the Americas during the age of exploration in the 16^{th} and 17^{th} centuries (*23*). The three major peach producing states in the United States are California (80%), Georgia, and South Carolina (*23*). The physical attributes of the peach can change from year to year depending on the weather; therefore many of the characteristics' defining peach cultivars may overlap. The fruit itself is a stone-fruit, usually circular in shape and covered with a thin layer of skin that often has a fuzzy appearance (*24*). The cultivars used for this study were Rich Lady and Crimson Lady which have a yellow skin color with a strong red blush overlay (*25-27*). Both are clingstone, yellow fleshed peaches. Depending on climate and cultivar, peach harvest can occur from late May into August.

Similar to peaches, the country of origin for Asian plums (*Prunus salicina* and related species*)* is China, which is still the major producer today supplying nearly half of the worlds plum supply (*28*). Plums are one of the most taxonomically diverse of the stone fruits and have adapted in many climate regions. The cultivar used in this study is Black Splendor plum; the fruit has black colored skin with beet-red flesh and is larger than an average plum. This particular cultivar ripens in early June (*29*).

Peach and plum cultivars contain a significant amount of phenolic compounds that are known to have powerful antioxidant capabilities, but for these stone fruits the bioactivity regarding inflammation has not been researched in depth (*13, 30*). The most

prevalent secondary plant compounds to focus on for stone fruits are phenolic acids, anthocyanins, and flavonoids (Table 2.1).

Table 2.1 Prevalent phenolic compounds, sorted by class, in peach and plum fruits.

Phenolic Acids (*30*)	caffeic acid, ferulic acid, sinaptic acid, vanillic acid, p-coumaric acid, chlorogenic acid, neochlorogenic acid, p-hydroxy benzoic acid, syringic acid
Anthocyanins(*31*)	cyanidin 3-glucoside, cyanidin 3-rutinoside
Flavonoids (*13, 31*)	catechin, epicatechin, quercetin, quercetin glycosides

Phenolic acids, namely hydroxycinnamic and hydroxybenzoic acids, are a major class of phenolic compounds found in foods of plant origin (Figure 2.3B). Hydroxycinnamic acids are generally esterified with quinic acid or glucose. For example, chlorogenic acid is commonly found in peach and plum fruits. This compound is structurally defined as the hydroxycinnamic acid, caffeic acid, esterified to quinic acid. Caffeic acid is listed as the predominant monomeric phenolic acid in each the peach and plum fruit, however most phenolic acids identified were in soluble form (chlorogenic acid isomers) (*30*). It has been reported that the serum concentrations of phenolics after consumption of a phenolic-rich meal were in the range of 0.1-10μM. This study further reported the percent absorption of caffeic acid to be 8.6-12% while that of chlorogenic acid is merely 0.3-2% (*32*). The mechanism of absorption of hydroxycinnamic acids is still unclear, so the reason for lower absorption of chlorogenic acid can only be estimated. Lower bioavailability may be due to limited absorptive capability, interaction of compounds within the food matrix, or quick elimination of dietary phenolics. A study investigating the bioavailability of chlorogenic acid and caffeic acid found results indicating they probable hydrolysis of chlorogenic acid into caffeic acid and ferulic acid after consumption (*33*). This would indicate a fruit high in

chlorogenic acid, such as peaches and plums, may still have a high absorption rate of hydroxycinnamic acids due to their hydrolyzation into monomeric form. Flavonoids are also often found in foods of plant origin and are divided into sub-classes - flavone, flavonol, flavanone, flavanonol, flavanols and anthocyanidins (*34*). The predominant flavonoid compounds in peach and plum fruits are flavanols (catechin, epicatechin) and flavon-3-ols (quercetin). Anthocyanidins are the glycoside-free match of the corresponding anthocyanin. Anthocyanins are often quantified as cyanidin 3-glucoside equivalents, as that is the main anthocyanin, with trace quantities of other glycosides (*13, 35*). Factors determining flavon-3-ol resistance to oxidation was proposed to be driven by the position of the hydroxyl groups in relationship to other functional groups, specifically the hydroxyl on carbon 3 in the C-ring (Figure 2.3B). Furthermore, unsaturation of the C-ring is important for increased antioxidant activity because it allows for electron delocalization to stabilize the ring (Figure 2.3A).

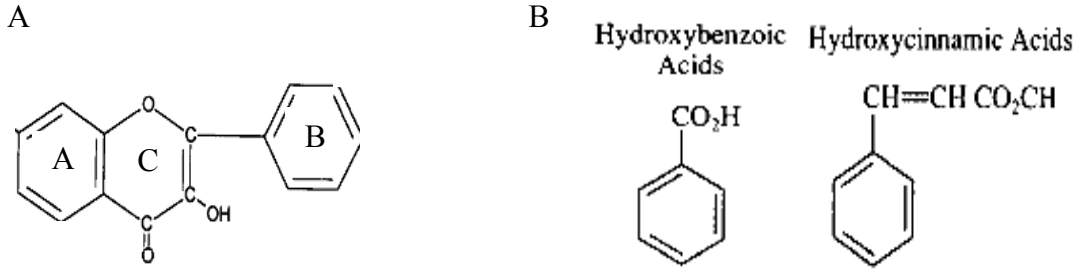


Figure 2.3 (A) General structure of flavon-3-ol and (B) the general structure of phenolic acids (hydroxybenzoic acid and hydroxycinnamic acids) (*34*).

When considering extraction and fractionation methods of phenolics it is important to determine the levels of soluble and insoluble phenolics - if a significant portion is insoluble a hydrolysis step may be needed. Studies indicate that in both peach and plum fruits, 90% of the total phenolic acids are soluble phenolics (*30*). For this

reason, it seems most common in the literature to use a methanol extraction to obtain the total soluble phenolics and assume a representative fruit extract.

There have been several in-vitro and in-vivo studies that have demonstrated the health benefits provided by phenolics found in peach and plum fruits, however there is limited research in discovering the anti-inflammatory potential of the secondary plant compounds directly from peach and plum fruits (*36-38*). These fruits contain a significant amount of bioactive compounds in their flesh and skin which have been shown to have high antioxidant properties (*13, 28, 30, 31, 35, 39-41*). A recent study investigated a large variety of peach and plum fruits at Texas A&M University to compare the antioxidant properties (*13*). The results indicated high antioxidant capacity of the cultivars, meriting a need for peach and plum fruits to be researched further to determine the potential health benefits. A follow up study identified chemopreventive activity of peach and plum extracts towards breast cancer cells (*14*). Based on recent literature, peach and plum fruits are suggestive of anti-inflammatory activity based on phenolic composition, however the anti-inflammatory activity targeting CVD related mechanisms have not been investigated.

2.6. General information of exotic fruits

The following is a brief overview of the selected fruit for this study describing the horticultural background, secondary plant compound composition, and potential bioactivities – according to published literature.

2.6.1. Pomegranate

The pomegranate fruit (*Punica granatum L.*) is grown in the Mediterranean regions, China, India, and the US (California and Arizona). While the pomegranate fruit has been researched in depth, there has been a recent growth in research dedicated to pomegranate juice. The fruit contains arils which surround the seed and contain small amounts of tart red juice. The arils are separated by a white, membranous pericarp which is generally viewed as inedible, however it is sometimes used as part of an extract for

dietary supplements (*42*). The edible part of the fruit is the arils which contain about 80% juice and 20% seed (*43*). Pomegranate have traditionally been used as an ancient form of medicine, but are currently being researched for the potential health benefits such as antioxidant activity, anti-inflammatory activity, and anticarcinogenic mechanisms.

The most therapeutically relevant compounds in pomegranates are ellagic acid ellagitannins, flavonoids, and anthocyanins (*42*). Synergistic effects of the aforementioned compound in pomegranates were studied by Lansky et al. The results suggested the effects of the compounds together were of greater benefit than the effects of ellagic acid alone (*44, 45*). The components of a pomegranate (seed, juice, and pericarp) contain very different phytochemistry. The seed is comprised of 12-20% oil, by weight, of which 99% are triacylglycerols and minor components of the oil are sterols, steroids, hydroxycinnamic acids, and antioxidant lignin derivatives (*46*). The juice, a bright red color, is made of mostly anthocyanins (3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin). These anthocyanins increase intensity during ripening and are reported to have strong antioxidant capabilities (*47*). The pericarp has a high presence of flavonoids and ellagitannins (*46*) such as punicalins and punicalagins. The ellagitannins found in pomegranates have been reported to be potential contributors to the antioxidant activity (*48*), however they are very large molecular weight compounds (up to 1084 g/mol) and they bioavailability has been questioned. The bioavailability of punicalagin was studied by Cerda et.al. through HPLC-MS-MS analysis of punicalagin metabolites following daily consumption of a supplement (0.6 – 1.2g) by rats (*49*). Results indicated a 3-6% absorption rate of the ingested punicalagin, which is an average absorption rate based on other phenolics (*32*). During postharvest many of these compounds are susceptible to quality loss due to several physiological and enzymatic disorders which can alter their beneficial health effects. Many of these postharvest issues are due to water loss during storage, leading to browning in both peel and arils. A study by Mirhedghan investigated the effect of a pre-storage heat treatment on the nutritive and functional properties during storage (*43*).

Heat-treated pomegranates contained higher levels of total phenolic compounds, total anthocyanins, sugars, and organic acids compared to those that were not heat-treated. This method can be considered a non-contaminant postharvest tool which prevents the degradation of beneficial compounds in pomegranate. A physiochemical study determined the differences in physical and chemical properties in pomegranates at unripe, half-ripe, and full-ripe stages. Results indicated significant differences in total weight, seed content, and densities of the fruit, however there was not a significant difference in phenolic content (*50*).

Although pomegranates have been consumed safely for many years, studies were done to ensure the safety for today's consumers. Initial studies of pomegranate fruit in animals at concentrations generally used in traditional medicine showed no toxic effects in terms of food intake, weight gain, behavioral or biochemical parameters (*51*). It has been previously found that punicalagin had possible toxic effects in cattle. A study evaluated the toxic effects of punicalagin in rats by providing a 6 % punicalagin-containing diet for 37 days. Results indicated non-significant decreases in food intake, food utility index, and growth rate during the first 15 days. The adverse effects were most likely due to the lower nutritional value of the punicalagin-enriched diet together with a decrease in its palatability (*49*). In a human clinical trial involving 64 overweight volunteers, the toxicity of a pomegranate (ellagitannin-enriched) polyphenol dietary supplement was evaluated. The subjects ingested amounts up to 1,420 mg/day (870 mg of gallic acid equivalents) for 28 days and upon consideration of results it was determined there was no toxic side effects of the pomegranate dietary supplement (*52*).

Pomegranates are reported to have antioxidant, anticarcinogenic, and anti-inflammatory properties likely because of the reported ability to scavenge free-radicals and decrease macrophage oxidative stress and lipid peroxidation (*42*). Pomegranates have also indicated a protective effect by increasing the plasma antioxidant capacity (*53*). A study by Schubert reported the antioxidant capabilities of fermented pomegranate juice to elicit greater antioxidant activity than red wine (*54*). A majority of the studies investigating the anticarcinogenic effectiveness of pomegranates utilized

cancer cell lines to test the potency of the extract against the cancer cells. The effects of fermented pomegranate juice and pericarp extract was investigated by challenging human prostate cancer cells (LNCaP, PC-3, and DU 145) with the extracts. The pericarp extract (70μg/mL) elicited a 50% decrease in proliferation of LNCaP due to the effect on cell cycle kinetics and induction of apoptosis. These results suggest significant antitumor activity in pomegranate extracts (*38*). Furthermore, a study designed to investigate prostate cancer found similr results. Results indicated mice challenged with prostate cancer that were pretreated with pomegranate extract showed a decrease in cancer cell proliferation as well as induction of apoptosis (*55*). Further clinical trials showed a decrease in serum prostate specific antigen upon the intake of eight ounces of pomegranate juice by 46 men with recurring prostate cancer. This trial included *in-vitro* assays indicating a 12% decrease in cancer cell growth and a 17% increase in apoptosis (*56*). Pomegranate juice consumption by patients with high blood pressure seemed to inhibit serum angiotensin converting enzyme (36% decrease) which, in turn, decreases the severity of vasoconstriction and reduce systolic blood pressure (5% decrease), thereby decreasing risk of CVD (*57*).

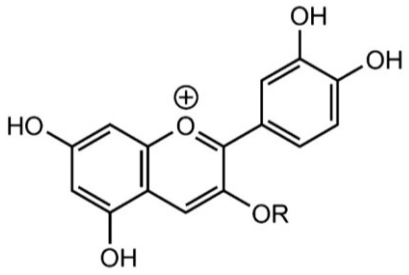
2.6.2. Açai

Açai (*Euterpe oleracea*, E. *precatoria* and E. *edulis*) is a slender, monoecious palm native to the Amazon estuary and has received much attention due to the secondary plant compound composition and the subsequent health benefits (*36, 58*). The fruit is dark purple in color, rounded and generally about 1cm in diameter. Common harvest season in Brazil is July to December. The fruit is often used to make beverages which are most popular in Brazil, Colombia, and Suriname (*58, 59*). In the Amazon, açai is consumed as a viscous pulp for medicinal use as an antidiarrheal treatment (*37*). Açai has been reported to have high antioxidant activity, a vasodilatory effect, and inhibition of inflammatory mechanisms (*59*).

Açai has recently come to international interest, being distributed world-wide as a functional food ingredient. However, international distribution is an issue due to the

instability of the easily degradable phenolic compounds, specifically anthocyanins. Açai pulp was reported to contain 282-303mg/100g of total anthocyanins, the two major compounds being cyanidin 3-glucoside and cyanidin 3-rutinoside (Figure 2.4) (*58, 60*). Individual quantifications of 202.3 ± 5.77mg/L cyanidin 3-glucoside and 75.1 ± 4.76 mg/L cyanidin 3-rutinoside were identified by Pacheco-Palencia (*58*). The anthocyanins found in açai have shown to be much less stable than anthocyanins found in other fruits (*58*). A study investigated the effect of storage time and temperature on the phenolics in açai and found the maximum loss of soluble phenolics at 30 days was 8% at 4ºC and 13% at 20ºC (*58*). In the same study the effect of clarification and ascorbic acid addition was also investigated. Results indicated the clarification process removed lipids and insoluble solids for aesthetic purposes. Ascorbic acid fortification, added to slow the browning process, induced anthocyanin polymerization causing difficulty in retaining antioxidant compounds in clarified juice. The clarification process had an initial detrimental effect on antioxidant capacity because polymeric anthocyanins were removed from the juice as well. Aside from being rich in anthocyanins, the açai fruit contains a multitude of other phenolic compounds. Pacheco-Palencia also identified (-)-epicatechin, (+)-catechin, four flavon-3-ol derivatives, and five phenolic acids (*58*). These results agree with previously reported literature by Lichtenthaler et al (*61*) and Del Pozo et al.(*62*). A review by Rice-Evans investigated the relationship between the structure of flavonoids and phenolic acids and their antioxidant activity (*34*). The review focused on the structure and how the hydrogen donating free radical scavenging mechanism was affected. Factors determining flavon-3-ol resistance to oxidation was proposed to be driven by the position of the hydroxyl groups in relationship to other hydroxyls, specifically they hydroxyl on carbon 3 in the C-ring (Figure 2.3A). Furthermore, unsaturation of the C-ring is important for increased antioxidant activity because it allows for electron delocalization to stabilize the ring (Figure 2.3A). Similar conclusions were drawn in reviewing the structure of phenolic acids. The basic structure consists of a benzene ring with a carboxylic acid functional group attached. Depending on the derivation of the compound, there may be a carbon chain connecting the benzene

ring to the carboxylic acid (Figure 2.3B). The review stated antioxidant activity was affected by the presence of a hydroxyl or other functional group attached to the benzene ring. Steric hindrance is the main determinant of antioxidant activity. The electron withdrawing properties of the carboxylic acid functional group has a negative influence on the hydrogen donating ability of the hydroxy benzoates, suggesting hydroxylated cinnamates to be more effective. These compounds are commonly found in fruits and have been researched in depth. Previous literature would suggest these compounds to elicit high antioxidant activity as well as overall health benefits (*30, 34, 36, 63*).



Cyanidin 3- glucoside; R = glucose

Cyanidin 3- rutinoside; R = rutinose

Figure 2.4 Structure of cyanidin, the predominant anthocyanin found in açai.

Within the past decade public interest in açai has led to many studies investigating the health benefits. A study designed to evaluate the pharmacokinetics of anthocyanins in açai pulp and juice determined that the plasma antioxidant capacity was significantly increased by up to 2.3 and 3 fold, respectively (*37*). These results suggested the bioavailability of anthocyanins from açai by eliciting an increase in plasma antioxidant capacity. An *in-vitro* study used a Caco-2 intestinal cell monolayer to determine if the absorption of açai polyphenolics was proficient . Results indicated that phenolic acids and monomeric flavonols in the presence of DMSO were readily transported from the apical to the basolateral side, suggesting a strong absorptive ability

(*36*). The *in-vitro* antioxidant capability of açai was investigated by measuring the inhibition of reactive oxygen species formation in neutrophils. Results indicated significant oxygen quenching suggesting antioxidants in açai are capable of entering human cells in a functional form. This study also determined the inhibitory effects of treatment with açai fruit on inflammatory mediators (*64*). Vasodilator potential of açai skin and seed extracts was determined by induction of vascular constriction followed by treatment with açai extracts. Results indicated the açai extracts induced endothelium dependent vasodilatation (*65*). Anticarcinogenic effects were also investigated by determining the anti-proliferative activity and induction of apoptosis with treatment of açai polyphenols on HL-60 human leukemia cells. Results showed a reduction in cell proliferation likely due to apoptosis via caspases-3 activation (*63*). These studies suggest açai is a good source of bioactive compounds that are highly active *in-vitro.*

2.6.3. Goji

The goji berry is grown from the plant *Lycium barbarum* and can be identified as *Fructis lychii,* wolfberry, and Kei Tze (*66*). The small red-orange berry was traditionally used in Chinese herbal medicine, and has been utilized in Korea and Japan cultures as well for over 250 years (*67*). The goji berry has historically been used for its anti-aging and anti-inflammatory effects (*68*). The berry has become popular in the media in the past decade, with claims of anticarcinogenic and increasing life expectancy. Currently, the goji berry is being studied in-depth, in efforts to find scientific backing to these claims. The goji fruit can be commercially purchased dried, as a juice, or in supplement form.

Previous literature has stated human consumption of the goji berry is acceptable and that toxicity is not an issue. The goji berry is a member of the Solanaceae family, which is known to contain toxic compounds, such as alkaloids. Consumption of alkaloids has been reported to lead to vomiting, diarrhea, pupil dilation and circulatory problems. Plants of the Solanaceae family have been reported to contain the alkaloid, atropine, which may affect muscle and nerve function and lead to fatality in high

concentrations. There have been reports of high levels (0.95%) of atropine found in dried goji berry grow in India. Upon further evaluation of goji berries from China and Thailand, only trace levels of atropine (19ppb) were isolated and the toxicity at such low levels is not a concern (*69*). A study screening the chemical and biological effects of Chinese herbal medicines investigated levels of the trace elements, arsenic and antimony, which are potentially toxic to humans. Results found the levels of these toxic metals to be low and not likely to be hazardous to human health (*70*). Finally, in a study investigating the bioavailability of zeaxanthin, it was claimed there were no published reports that describe toxic effects due to the consumption of the goji berry (*66*). The fruit has been consumed by humans for an extended period of time and no studies have reported any adverse effects of the goji berry, isolates or concentrates.

The secondary plant compound composition of the goji berry has been studied in-depth. The goji berry contains unique polysaccharide compounds that are reported to be related to the bioactivity of the fruit (*68, 71-73*). There are five polysaccharides-protein complexes (LbGl1-LbGp5), however these have been poorly characterized, chemically. A study designed to investigate the glycan structure of the protein-polysaccharides found a 90.7% carbohydrate content which was composed of arabinose, galactose, and amino acids (*74*). These complexes protect polyunsaturated fatty acids against lipid peroxidation and are reported to have a number of health benefits (*72, 73*). Goji berries also contain a significant level of carotenoids which comprise 0.03 to 0.5% dry weight of the fruit. Beta-carotene and zeaxanthin were identified, while the most prominent carotenoid found was zeaxanthin dipalmitate at 31-56% of the total weight of carotenoids (*75*). The high level of carotenoids found in the berry is what generates the characteristic orange color. The goji berry does not contain high concentrations of ascorbic acid, however a recent study found a glycosylated form of ascorbic acid present at about 0.5% of the fruit (dry weight) (*76*). Other reported secondary plant compounds include p-coumaric acid, chlorogenic acid, betaine, hydroxycinnamates, benzoates, and trace amounts of anthocyanins. However, the reported bioactive compounds have been reported to be the carotenoids, betaine, and the polysaccharide protein complexes (*66-*

68, 71, 72, 75, 77, 78). The secondary plant compound composition of the goji berry reports compounds that are well known for their antioxidant activity, suggesting this fruit to potentially elicit high bioactivity *in-vitro*.

Due to the unique phytochemistry of the goji berry, there is a plethora of research investigating the health benefits of this fruit. The polysaccharide-protein complexes are widely researched and were reported to be the active therapeutic compound from the goji berry (*78*). The results of a study investigating immunomodulation and antitumor activity indicated tumor growth inhibition and an improvement in immune system function, although the effect was not dose dependent. The optimal treatment dose reported was 10 mg/kg, which showed a higher effect than 5 and 20 mg/kg doses (*71*). A series of investigations tested the effect of protein polysaccharides on human skin indicating potential skin-protective properties. A study by Wang et al. induced oxidative stress (promoting lipid peroxidation and cytochrome C reduction via free radicals) to evaluate the effects of goji berry extracts using a testicular tissue culture system in a hypothermic state. Results indicated the protein polysaccharide fraction was a potent inhibitor of lipid peroxidation and cytochrome C reduction, thereby protecting the seminiferous epithelium from structural damage and apoptosis (*73*).These results led to further investigation of the protein polysaccharides as a protective skin agent. Results indicated promoted survival of skin fibroblasts cultured in suboptimal conditions, likely due to the high anti-apoptotic and antioxidant compounds present in the extract (*78*). As discussed previously, goji berries are rich in the carotenoids, specifically zeaxanthin dipalmitate (*75*). Zeaxanthin has been suggestive of preventing age-related macular degeneration. Zeaxanthin accumulates on the macula of the eye, providing antioxidant and blue-light absorbing properties. A human intervention trial evaluated the change in plasma zeaxanthin concentrations after dietary supplementation with whole goji berries. Zeaxanthin dipalmitate is structurally similar to zeaxanthin, as it is zeaxanthin esterified to 2 fatty acid chains (palmitic acid) (Figure 2.5).

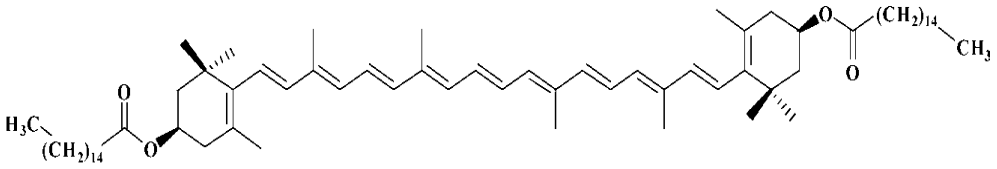


Figure 2.5. Chemical Structure of zeaxanthin dipalmitate (*75*).

The treatment group consumed 15 grams/day of goji berries (about 3mg zeaxanthin) for 28 days which lead to a 2.5 fold increase in plasma concentration indicating its apparent bioavailability and potential health benefits, specifically towards macular degeneration. (*66*). Zeaxanthin is absorbed in the small intestine without the esterified fatty acid, so hydrolysis in the small intestine is crucial to bioavailability. A bioavailability study investigated potential bioavailability enhancements of zeaxanthin. A dose of 15mg of zeaxanthin was consumed with a standardized breakfast in healthy human subjects. Blood samples were collected immediately before and up to 10 h post-injection ad measured by HPLC. Results showed zeaxanthin peaked at 6 h post-ingestion and indicated a 3-fold enhancement of bioavailability when berries were homogenized in hot (80°C) skimmed milk, as opposed to warm (40°C) skimmed milk and hot (80°C) water (*79*). The researchers suggested that the higher processing temperature in congruence with the milk proteins enhanced the zeaxanthin bioavailability of goji berry by possibly improving its incorporation into mixed micelles, uptake, and release in triacylglycerol-rich lipoproteins. The exact mechanism, however, is still unclear. There has been much investigation in determining the secondary plant compound composition and their related health benefits. Much of the research has been related to antioxidant and anticarcinogenic activities, however there is little to no research investigating the anti-inflammatory activity of the fruit relating to prevention of cardiovascular disease.

3. PEACH AND PLUM PHENOLICS DECREASE OXIDATIVE STRESS AND INFLAMMATION IN VASCULAR ENDOTHELIAL CELLS

3.1. Introduction

Inflammation and oxidative stress have been a growing research interest due to their correlation with Metabolic Syndrome (*80, 81*). Metabolic Syndrome is defined by a combination of medical disorders including abdominal obesity, hypertriglyceridemia and hypertension, which put one at increased risk for cardiovascular disease (CVD) (*1, 81*). CVD is the leading cause of mortality in the United States (*2, 8*) and is defined as diseases of the heart involving the vasculature (arteries and veins). Vascular disorders, through over-expression of adhesion molecules and cytokines (leading to inflammation), are thought to participate in the pathogenesis of atherosclerosis, a common manifestation of CVD (*2, 12*). CVD is the leading cause of mortality in the United States (*2, 8*).

A recent study investigated a large variety of peach and plum fruits at Texas A&M University to compare the antioxidant properties (*13*). These fruits contained a significant amount of bioactive compounds in their flesh and skin which have been shown to have high antioxidant properties (*13, 28, 30, 31, 35, 39-41*). These results merit a need for peach and plum fruits to be researched further and determine the potential health benefits. The purpose of this study is to investigate the antioxidant and anti-inflammatory effects of the total extract, as well as the anthocyanin and non-anthocyanin phenolic (NAP) fractions, of peach and plum fruits; specifically targeting mitigation of oxidative stress and inflammatory pathways through disruption of NF-κB mechanisms. Understanding the phenolic composition and biological activity of these fruits would increase the potential for use in the food, beverage, and health related industries.

3.1.1. Potential anti-inflammatory effects on the NF-κB mediated inflammatory pathway

Inflammation is an organism's defense against harmful pathogens or physical injury. On tissue-level, inflammation is an influx of leukocytes to the site of the pathogen, disrupting the integrity of the endothelial layer (Figure 2.1). Inflammatory

cytokines, oxidative stress, vascular stress, and shear forces are the most prominent stimuli of inflammation leading to activation of the specific toll-like receptor (TLR-4). A signal transduction pathway involving signaling proteins IRAK-1 and TRAF-6 is activated leading to phosphorylation of the inhibitor-bound NF-κB, a ubiquitous transcription factor. NF-κB is located in the cytoplasm bound to an inhibitor molecule. Upon degradation of the inhibitor, translocation to the nucleus occurs. Activation of NF-κB results in subsequent changes in gene expression mediating the production of multiple cytokines (Interlukin-6 and Interlukin-8), enzymes (COX-2), and cell adhesion molecules (V-CAM and I-CAM). These are important for early atherosclerotic lesion formation (*17, 21*) (Figure 2.1 and 2.2).

Oxidative stress and inflammatory mechanisms are reported to be interconnected, as oxidized-LDL has been suggested to play a role in progression of early inflammatory lesions. Intracellular cell adhesion molecules (I-CAM-1) and vascular cell adhesion molecules (V-CAM-1) mediate the adhesion of monocytes to the vascular endothelium which then enters the sub-endothelial space, further differentiating into a macrophage. Macrophages accumulate oxidized low-density lipoproteins (LDL) via endocytosis to become foam cells which aggregate, causing endothelial dysfunction and tissue injury (Figure 2.1). Macrophages also generate reactive oxygen species (ROS) leading to an environment of oxidative stress. This cyclic mechanism implies that antioxidants can potentially decrease ROS levels and disrupt the inflammatory pathway which leads to genesis of vascular lesions (*16*).

By analyzing mRNA of LPS-challenged HUVEC cells with and without treatment of fruit, the gene expression of pro-inflammatory markers can be determined. The expression of specific mRNA coding for pro-inflammatory biomarkers is measured to determine if there has been an effect on the inflammatory pathway.

3.1.2. Secondary plant compound composition of stone fruits

It is well established that peach and plum cultivars contain a great amount of phenolic compounds that are known to have powerful antioxidant capabilities, but the

inflammatory mechanisms have not been researched in depth (*13, 30*). Peach fruits contain mainly the phenolic acids: caffeic acid, chlorogenic acid and chlorogenic acid derivatives; the flavan-3-ols: catechin, epicatechin, and their respective derivatives; the flavonols: quercetin and quercetin derivatives; and a small amount of the anthocyanin: cyanidin glycosides (*31*). The plum fruit contains a similar phenolic profile with a much higher concentration of anthocyanins (*13, 31*).

There have been several *in-vitro* and *in-vivo* studies that have demonstrated the health benefits provided by these phenolics, however there is limited research in discovering the anti-inflammatory potential of the secondary plant compounds extracted directly from peach and plum fruits (*36-38*). The positive results of previous research merits this investigation of the anti-inflammatory and antioxidant effects of peach and plum fruits.

3.2. Materials and methods

3.2.1. Sourcing

″Black Splendor″ (BS), a commercial variety of red-fleshed plums (*Prunus salicina*) was collected at a mature, firm stage. This is true for "Rich Lady" (RL) and "Crimson Lady" (CL) yellow-fleshed peaches as well. These were obtained from commercial packing houses in California near Fresno, shipped by overnight transport to the Department of Horticultural Sciences at Texas A&M University (College Station, TX), and stored in the cold room (4C) until processing. The fruit was processed by removing the seed and storing at -80 °C until use.

3.2.2. Phenolic extraction for secondary plant compound and in-vitro analysis

The fruit was extracted in 100% methanol. Crude extract was fractionated into total, anthocyanins, and non-anthocyanin polyphenolics (NAP) fractions by solid phase extraction using a C18 cartridge. The extract was loaded into the SEP Pack C18 cartridge conditioned with 100% methanol and nanopure water. After sample addition, the cartridge was washed with nanopure water to remove free sugars. The NAP fraction

containing a mixture of phenolic acids and flavonols/procyanidins was eluted using ethyl acetate. Acidified methanol (0.01% HCl) was then added to elute the anthocyanin fraction. The total extract was obtained by loading the extract onto a SEP Pack C18 cartridge preconditioned with methanol and water and eluted with acidified methanol (0.01% HCl). Extracts were filtered and used directly for spectrophotometric HPLC-PDA.

The same extraction and fractionation method for secondary plant compound analysis was used for *in-vitro* analysis followed solvent removal under reduced pressure at 40°C and drying using a centrifugal evaporator. Extracts were re-dissolved in dimethyl sulfoxide (DMSO) to form concentrates. All extracts were normalized to the predetermined phenolic content based on cell viability for *in-vitro* assays, allowing the bioefficacy to be better ascribed to the occurrence of specific compounds.

3.2.3. HPLC-PDA and HPLC-ESI-MS

Separations will conducted on a Waters 2695 Alliance system and polyphenolics will be identified and quantified based on spectroscopic characteristics and retention time, as compared to authentic standards. Unidentified flavonoids will be quantified in rutin equivalents, procyanidin-based compounds will be expressed in (+)-catechin equivalents, and phenolic acids in gallic acid. Mass spectrometric analysis (via chromatographic separations and by direct infusion into the analyzer) will be performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. Electrospray ionization is conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V.

3.2.4. Total soluble phenolics

Folin-Ciocalteu assay was conducted to quantify total reducing equivalents (representative of total soluble phenolics) in gallic acid equivalents. 100µL sample was

added to 1mL 0.25N Folin-Ciocalteu reagent and set for 3 minutes. Then, 1mL 1N sodium carbonate was added and set for 7 minutes. Finally, 7.9mL water was added to the solution and set for 1 hour. The samples were read at 726nm using a BMG Labtech FLUOstar fluorescent microplate reader.

3.2.5. Oxygen radical absorbance capacity (ORAC)

ORAC measures the ability of antioxidants to inhibit the decline in fluorescence induced by action of the peroxy radical generator, AAPH. 50 µL treatments of peach and plum extract, 100µL flourescein, and 50µL AAPH solution were quickly injected into a 96 well plate. A standard curve of trolox (0-200µM) was used. The fluorescent activity was measured every 120 seconds for 90 minutes using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC) and expressed as trolox equivalents (TE).

3.2.6. Human umbilical vein endothelial cells (HUVEC)

HUVEC were cultured in endothelial cell medium (ECM) containing 5% fetal bovine serum, 1% of endothelial cell growth supplement 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37 °C and 5% CO2, and utilized between passages 4-9.

3.2.7. Cell viability

Cell viability was measured by using the Promega CellTiter 96® AQueous One Solution Cell Proliferation assay. HUVEC cells were seeded ($8x10^3$ cells/100µL) in costar tissue-culture treated 96 well plates and incubated for 24 hours at 37 °C and 5% CO2 to allow for attachment. HUVEC medium was removed, cells washed with phosphate buffered saline (PBS), and incubated with 100µL BS, CL, and RL extracts (in DMSO) for 24 hours at 37 °C and 5% CO2. The extract suspension was removed and the cells washed with PBS. A solution of 100µL HUVEC medium and 20µL CellTiter 96®

AQueous One Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was added to each well and incubated for two hours followed by an absorbance reading at 490nm using a BMG Labtech FLUOstar fluorescent microplate reader. Results were expressed in percent viability based on control cells, following the manufacturer's protocol.

3.2.8. Reactive oxygen species measurement

Intracellular ROS was estimated by following the method of Meng et al. using 2',7'-dichlorofluorescin diacetate (H_2DCF-DA) as a probe (*82*). Briefly, HUVEC was grown in 96-well plates ($7x10^4$ cell/100μL) and pretreated with BS, CL, or RL extracts at varying phenolic concentrations for 24hrs followed by exposure to 100μM H_2O_2 for 2hrs. Residual H_2O_2 was removed and cells washed using PBS followed by staining with 10μM H_2DCF-DA. HUVEC were stained at 37ºC for 30min as the fluorescence signal was monitored at 530nm with excitation at 488nm with BMG Labtech FLUOstar fluorescent microplate reader (BMG Labtech Inc., Durham, NC). Results are expressed in relative fluorescence units (RFU) relative to the control.

3.2.9. Real-time PCR analysis of messenger-RNAs

HUVEC cells (passage 4-9) were incubated in six-well culture plates at $1x10^5$ cell/ml for 24hr at 37 °C and 5% CO2, allowing for attachment. HUVEC medium was removed and cells washed with PBS, followed by a 30 minute pretreatment of varying BS, CL, or RL concentrations at 37 °C and 5% CO2. Cells were then challenged with 1μg/mL LPS for 3hr (NF-κB, TLR-4, IRAK-1, TRAF-6) or 24hr (V-CAM-1, COX-2, IL-6, IL-8) exposure periods at 37 °C and 5% CO2.

Total RNA was isolated using the mirVana™ miRNA Isolation Kit Applied Biosystems (AB, Foster City, Ca) following the manufacturer's recommended protocol. Samples were evaluated for quality and quantity using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR

according to the manufacturer's protocol. PCR was carried out with the SYBR® Green PCR Master Mix from Applied Biosystems, following manufacturer's protocol, on an ABI Prism 7700 Sequence Detection System (PE Applied). Primers encoding human IL-6, IL-8, ICAM-1, VCAM-1, IRAK-1, TRAF-6, COX-2, TLR-4 and NF-κB were purchased from Integrated DNA Technologies, Inc. (San Diego, CA) and for amplification. Product specificity was examined by dissociation curve analysis.

3.2.10. Statistical analysis

Quantitative data represented by mean values with the respective standard error of the mean corresponding to 3 or more replicates. Data was analyzed by one-way analysis of variance (ANOVA) using JMP version 5 (SAS Institute Inc.). Post-hoc Tukey pairwise comparisons were used ($p<0.05$).

3.3. Results and discussion

3.3.1. Secondary plant compound analysis

The total soluble phenolic (TSP) content was measured using the Folin-Ciocalteu assay and a correlation of TSP to fresh fruit weight was made. The secondary plant compounds in peach and plum fruits were analyzed using HPLC-Mass Spectrometry to determine structural features.

The phenolic content of peach and plum fruits was quantified using the Folin-Ciocalteu assay (Table 3.1). Black Splendor plum contains a much higher total soluble phenolic content (1.543 mg TSP/g fruit) comparatively to the peach cultivars. Rich Lady peach and Crimson Lady peach contained 0.164 mg TSP/g fruit and 0.246 mg TSP/g fruit. The anthocyanin fraction of red-fleshed BS plum (1.238 mg TSP/g fruit) was much more concentrated than the yellow-fleshed RL and CL peaches, containing 0.119 and 0.152 mg TSP/g fruit, respectively. CL peaches have a darker skin than RL peach, which may account for the slightly higher phenolic content in the anthocyanin fraction. RL peach was the only of the fruits to have the NAP fraction contain the highest phenolic

content. Both BS and CL contained a significantly lower NAP phenolic content compared to their total and anthocyanin fractions.

Table 3.1 Table represents the concentrations of phenolics in the total extract and each fraction. Data obtained using Folin-Ciocalteu method and results are expressed in gallic acid equivalents as mean ± standard error of the mean based on replicates, n ≥ 3.

Black Splendor Plum	***Rich Lady Peach***	***Crimson Lady Peach***
mg Phenolics/g fruit (FW)		
Total 1.543 ± 0.31	**Total** 0.164 ± 0.01	**Total** 0.246 ± 0.03
Anthocyanin 1.238 ± 0.13	**Anthocyanin** 0.119 ± 0.003	**Anthocyanin** 0.152 ± 0.05
NAP 0.521 ± 0.05	**NAP** 0.246 ± 0.03	**NAP** 0.088 ± 0.01

The combination of photo diode array (PDA) and electrospray ionization mass spectrometry (ESIMS) coupled to the HPLC equipment provided an accurate method for identification of individual phenolics. The methanol extracts were separated by reverse phase HPLC followed by identification of compounds based on retention time from UV spectra and the fragmentation pattern from compound ionization using the ESIMS data. The HPLC-PDA analysis showed the procyanidins had a low response factor, making identification of the compounds difficult. The response factors for hydroxycinnamates, flavonoids and anthocyanins were quite good, particularly in the specific wavelength of each compound type (hydroxycinnamates and flavonoids– 360nm; anthocyanins – 520nm). The separation of the procyanidin compounds, seen through HPLC-PDA, was not ideal, and was often co-eluted with closely-eluting compounds. The HPLC-ESIMS analysis indicated that in the case of procyanidins, the dimeric flavan-3-ols ionized better than the monomers (catechin and epicatechin).

Table 3.2. HPLC-PDA and HPLC-ESIMS of stone fruit phenolics.

Phenolic	*No.*	*Rt HPLC (min)*	*HPLC-UV-PDA (nm)*	*HPLC-ESIMS (m/z)*
		Anthocyanins		
Cyanidin 3-glycoside	17	23.4	519	447
Cyanidin 3-Rutinoside	1	24.27	519	593, 284
		Hydroxycinnamates		
Neochlorogenic Acid	2	25.96	325	353, 179
Chlorogenic Acid	16	29.81	325	353, 179
Caffeic Acid Derivative	5	33.83	325, 289sh	335, 179
		Flavonols		
Quercetin 3-rutinoside	7	40.83	355, 254	609, 301
Quercetin 3-glucoside	8	41.38	355, 254	463, 301
Quercetin 3-pentosyle-pentoside	9	42.42	355, 254	565, 300
Quercetin 3-xyloside	10	43.40	355, 254	433, 301
Quercetin 3-rhamnoside	11	44.24	355, 254	447,301
		Phenolic Acid		
Syringic Acid hexoside	6	38.11	280	359, 197
		Flavan 3-ols		
Procyanidin trimer	-	21.69 (BS)	280	865, 577
Procyanidin diner	13	22.02	280	577, 425, 289
Procyanidin B2	4	22.55	280	577, 425,407, 289
Procyanidin tetramer	18	23.17	280	1153, 865, 577, 425, 289
Procyanidin pentamer	-	24.54 (BS)	280	1441, 1153, 865
Catechin	14	25.98	280	335*
Procyanidin B2	-	24.83 (RL)	280	577, 425,407, 289
Procyanidin B2	-	26.02 (BS)	280	577, 425,407, 289
Procyanidin tetramer	3	27.3	280	1153, 865, 577, 425, 289
Epicatechin	12	30.45	280	289

* = possible formic acid adduct

Rich Lady and Crimson Lady peach total extracts showed nearly identical phenolic profiles (Figure 3.1). The chromatograms recorded at 280nm showed two main peaks indicating catechin (**14**) and chlorogenic acid (**16**). Chlorogenic was characterized through HLPC-ESIMS by the characteristic spectra (*m/z* 353 [M-H]+; *m/z* 179) (*31*). Catechin was characterized through HLPC-ESIMS by identification of *m/z* 289 [M-H]+, however chromatograms of both CL and RL indicated an *m/z* 335, which would suggest catechin with a formic acid adduct. The HPLC-ESIMS spectra of CL (Figure 3.1A) and RL (Figure 3.1B) indicated the presence of procyanidin derivatives; however the HPLC-PDA chromatogram did not show good separation of the compounds (Figure 3.1).

Procyanidin B2 (**4**) was identified by the characteristic HPLC-ESIMS spectra (*m/z* 577 [M-H]+; *m/z* 425 *retro*-Diels-Alder fragment; *m/z* 407; and *m/z* 289, fragments of terminal and remaining catechin residues) (*31*). A procyanidin dimer (**13**) and tetramer (**18**) (characteristic fragmentation, Table 3.2) (*83*) were also detected. Rich Lady peach indicated the presence of another procyanidin B2, although not fully identified in the HPLC-PDA chromatograms. HPLC-ESIMS suggested co-elution of the procyanidin derivatives (Rt = 21-26min) with the anthocyanin compound (Table 3.2). Flavonols were also detected in both CL (Figure 3.1A) and RL (Figure 3.1B) peach extracts; all detected compounds were quercetin derivatives. The two quercetin derivatives were identified at 280nm. The main peaks were quercetin 3-rutinoside (**7**) and 3-glucoside (**8**) (characteristic UV spectrum and MS fragments at *m/z* 301 and 300) (Table 3.2). The CL peach chromatograms of 510nm (spectra now shown) revealed the presence of cyanidin-3-rutinoside (**1**), identified by its characteristic HPLC-MS spectrum and fragments (*m/z* 593 [M-H]+; *m/z* 284) (Table 3.2). The RL peach chromatograms of 510nm (spectra now shown) revealed the presence of cyanidin (**17**), identified by its characteristic HPLC-MS parent ion (*m/z* 477 [M-H]+), however no fragmentation was seen (Table 3.2).

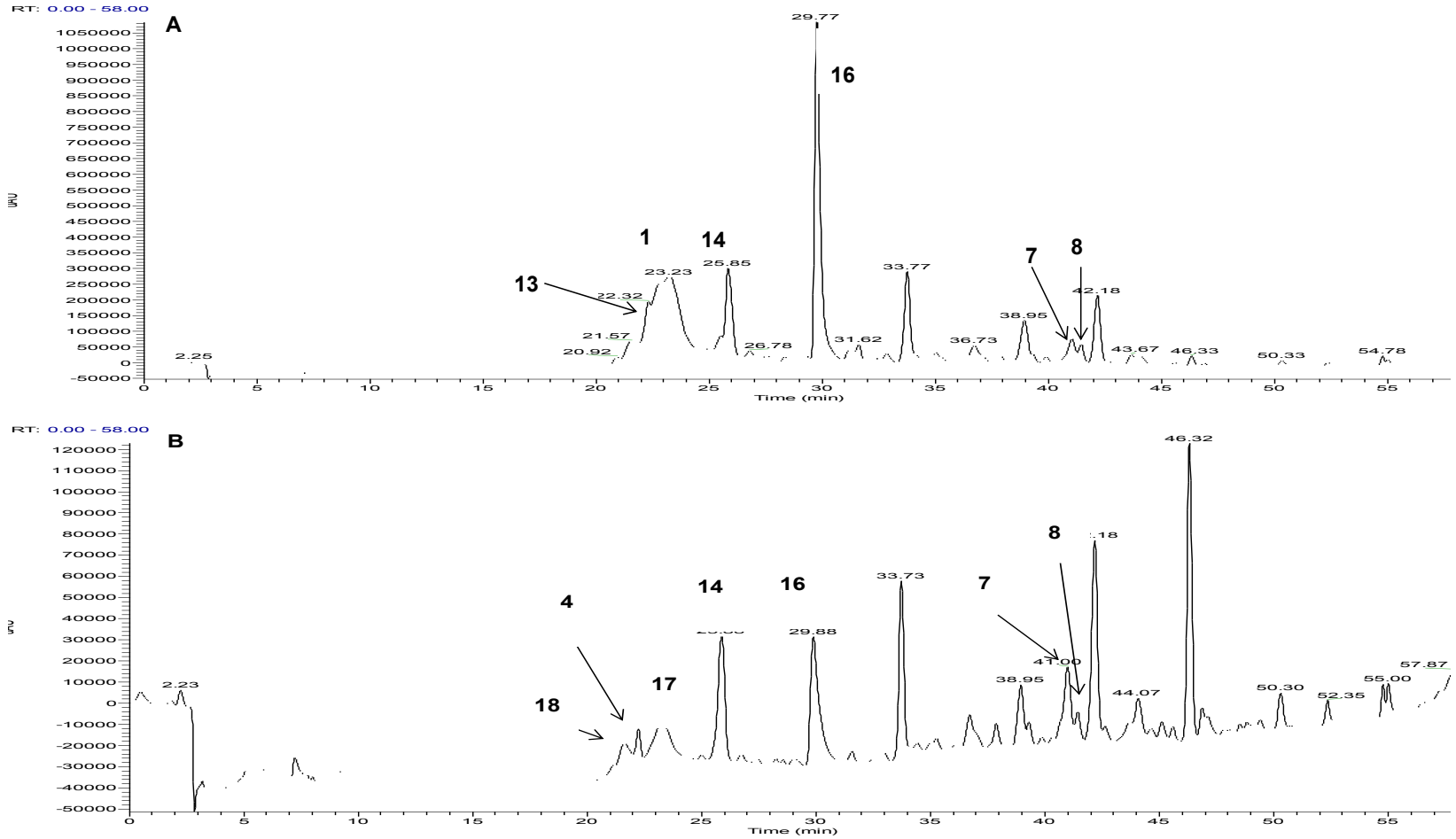


Figure 3.1 HPLC chromatogram of Crimson Lady peach total extract recorded at 280nm (A) and Rich Lady peach total extract recorded at 280nm (B). **(1)** Cyanidin 3-rutinoside; **(4)** Procyanidin B2; **(7)** Quercetin 3-rutinoside; **(8)** Quercetin 3-glucoside; **(13)** Procyanidin dimer; **(14)** Catechin; **(16)** Chlorogenic acid; **(17)** Cyanidin 3-glycoside; **(18)** Procyanidin tetramer.

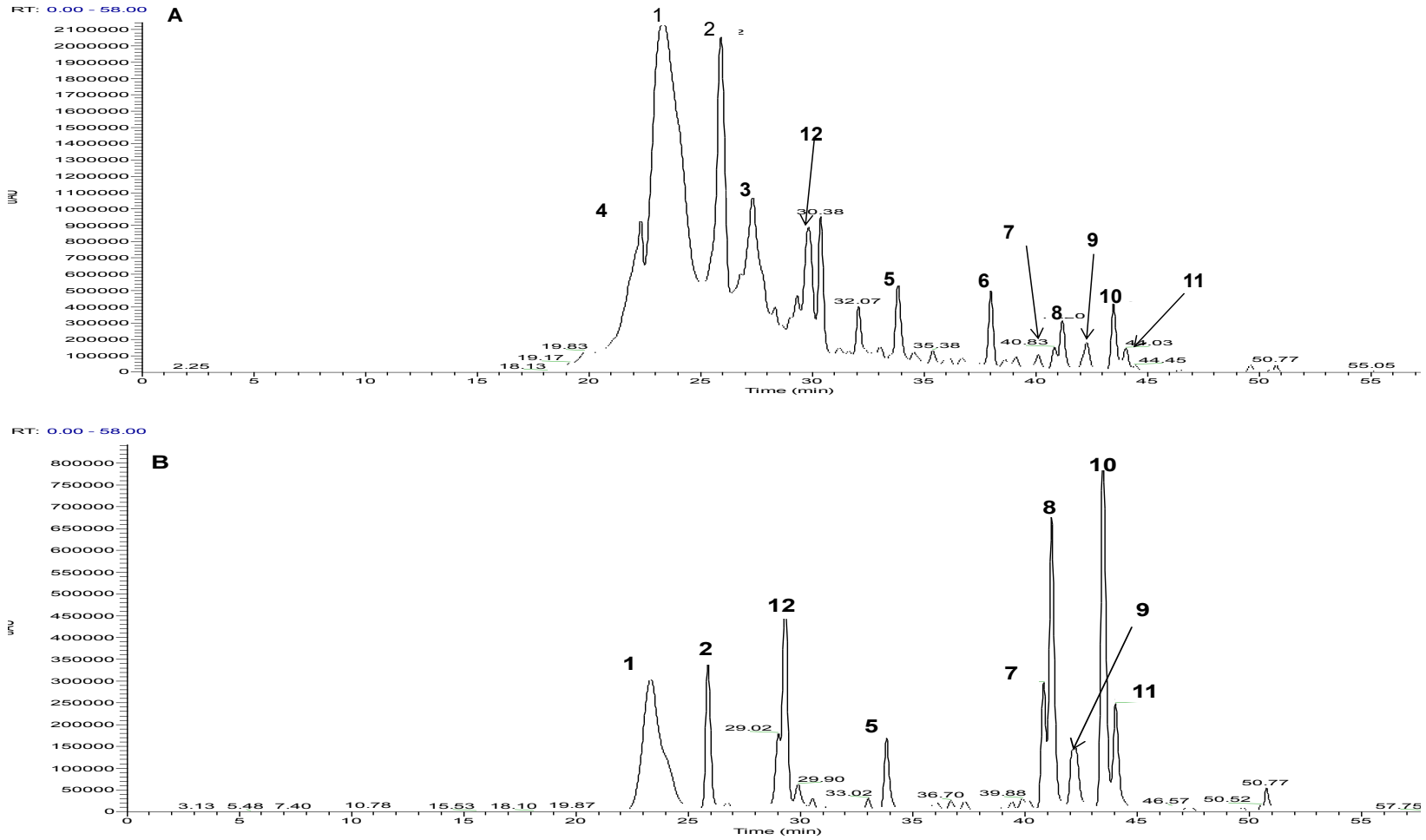


Figure 3.2 HPLC chromatogram of Black Splendor plum total extract recorded at 280nm (A) and 360nm (B). **(1)** Cyanidin 3-rutinoside; **(2)** Neochlorogenic acid; **(3)** Procyanidin tetramer; **(4)** Procyanidin B2; **(5)** Caffeic acid derivative; **(6)** syringic acid hexoside; **(7)** Quercetin 3-rutinoside; **(8)** Quercetin 3-glucoside; **(9)** Quercetin 3-pentosyl pentoside; **(10)** Quercetin 3-xyloside; **(11)** Quercetin 3-rhamnoside; **(12)** Epicatechin.

The HPLC chromatograms of BS plum was quite different from those of the peaches, however the secondary plant compound composition was similar (Figure 3.2). The BS plum extract contained an unidentified caffeic acid derivative (**5**) with a [M-H]+ ion at *m/z* 335 and the characteristic fragment of caffeic acid at *m/z* 179 (Table 3.2). HPLC-ESIMS also identified neochlorogenic acid (**2**) with a [M-H]+ ion at *m/z* 353 and the characteristic fragment of caffeic acid at *m/z* 179 (Table 3.2). Based on elution of chlorogenic acid in CL and RL peaches (Rt = 29.81 min), this compound was determined to be neochlorogenic because of the earlier elution time (Rt = 25.96 min) (Figure 3.2). In the 280 nm chromatogram and the HPLC-ESIMS spectra, the presence of procyanidin derivatives was also detected (Figure 3.2A). These were identified as the dimers procyanidin B2 (**4**) by their characteristic HPLC-ESIMS spectra (*m/z* 577 [M-H]+; *m/z* 425 *retro*-Diels-Alder fragment; *m/z* 407; and *m/z* 289, fragments of terminal and remaining catechin residues) (*31*). Another procyanidin B2 as well as a trimer and pentamer (characteristic fragmentation, Table 3.2) (*83*) were also detected through HPLC-MS, although not fully identified in the HPLC-PDA chromatograms. HPLC-MS suggested co-elution of the procyanidin derivatives (**3,4**) (Rt = 21-26min) with the anthocyanin compounds (Figure 3.2A). HPLC-ESIMS also identified epicatechin (**12**) with a [M-H]+ ion at *m/z* 289 (Table 3.2). Based on elution of catechin in CL and RL peaches (Rt = 25.98 min), this compound was determined to be epicatechin because of the later elution time (Rt = 30.45 min). Flavonols were also detected in the BS plum extract, all detected compound were quercetin derivatives (characteristic UV spectrum and MS fragments at *m/z* 301 and 300) (Table 3.2). Five quercetin derivatives were identified at 280nm (Figure 3.2A) and 360nm (Figure 3.2B). The main peaks were quercetin 3-xyloside (**10**) and 3-glucoside (**8**) plus minor amounts of quercetin 3-rutinoside (**7**), 3-pentosyl pentoside (**11**), and 3-rhamnoside (**9**) (Figure 3.2B). The chromatograms of 510nm (spectra not shown) revealed the presence of cyanidin-3-rutinoside (**1**), identified by its characteristic HPLC-MS spectrum and fragments (*m/z* 593 [M-H]+; *m/z* 284) (Table 3.2). HPLC-ESIMS also identified syringic acid hexoside

(**6**) with a [M-H]+ ion at *m/z* 359 and the characteristic fragment of caffeic acid at *m/z* 197 (Table 3.2).

In general, our findings regarding the phenolic profile of BS, RL, and CL extracts and fractions are in agreement with the previously reported data for peach and plum varieties (*31*). Hydroxycinnamic and hydroxybenzoic acids reported in peach and plum fruits are chlorogenic acid and neo-chlorogenic acid. There is little research on the effect of these compounds on inflammatory mechanisms, however they have been reported to have high antioxidant capacities (*30*). Flavonoids have long been reported as free-radical scavengers due to their unique structural composition. Peach and plum fruits are rich in the flavan-3-ols catechin and epicatechin, as well as in the flavonols quercetin glycosides (*31*). Phenolic acids have been reported to have high antioxidant activity, the predominant phenolic acid in both peach and plum fruits being caffeic acid, which is often derived through hydrolysis of chlorogenic acid (*28, 30, 34, 36*). Anthocyanins are present in both peach and plum fruits, however they are much more prominent in red-fleshed plums. Like flavonoids, anthocyanins are well known antioxidants, as they have been the subject of many studies in recent years (*13, 31, 35, 84*). The anthocyanins reported in peach and plum fruits are cyanidin 3-glycoside (*31*). These phenolics have been the subject of many health-related claims related to antioxidant and anti-inflammatory properties of the dietary compounds (*13, 30, 31, 35, 85*).

3.3.2. Fruit extracts treatment in-vitro and the corresponding fresh fruit weight

The viability of HUVEC cells treated with BS, CL, and RL extracts were monitored to ensure results of further assays would not be altered by cell death from toxicity of fruit extract. The cell proliferation assay is a colorimetric method which measures the activity of enzymes in metabolically active cells, determined by reduction of the CellTiter 96® AQ_{ueous} One Solution Reagent, essentially obtaining a viable cell count.

BS-Total and BS-Anthocyanin plum extracts did not significantly alter cell viability of HUVEC cells at concentrations within the range of 0-10 mg/L. At

concentrations greater than 10mg/L, HUVEC cells began to show a decrease in cell-viability due to a toxicity effect from the extracts. Concentrations of 0-10mg/L were chosen for use in *in-vitro* assays for BS-Total and BS-Anthocyanin plum extracts. BS-NAP plum increased cell growth up to 20mg/L, where cell-viability then became compromised (Figure 3.3A). BS-NAP plum concentrations of 0-15mg/L were selected for use in *in-vitro* assays.

CL and RL peach extracts, aside from RL-total, did not significantly alter cell viability of HUVEC cells at concentrations up to 20 mg/L. A decrease in cell-viability was seen at concentrations greater than 20mg/L (Figure 3.3B, 3.3C). Extract concentrations of 0-15mg/L were selected for the determination of antioxidant and anti-inflammatory effects in HUVEC cells for CL-Total, CL-Anthocyanin, CL-NAP, RL-Anthocyanin, and RL-NAP. Although results indicated little affect of cell viability at 0-15mg/L for RL-Total extract, preliminary results indicated cell death at 15mg/L with extract treatment followed by an LPS-challenge. For this reason, 10mg/L RL-Total and below were selected for use in *in-vitro* assays.

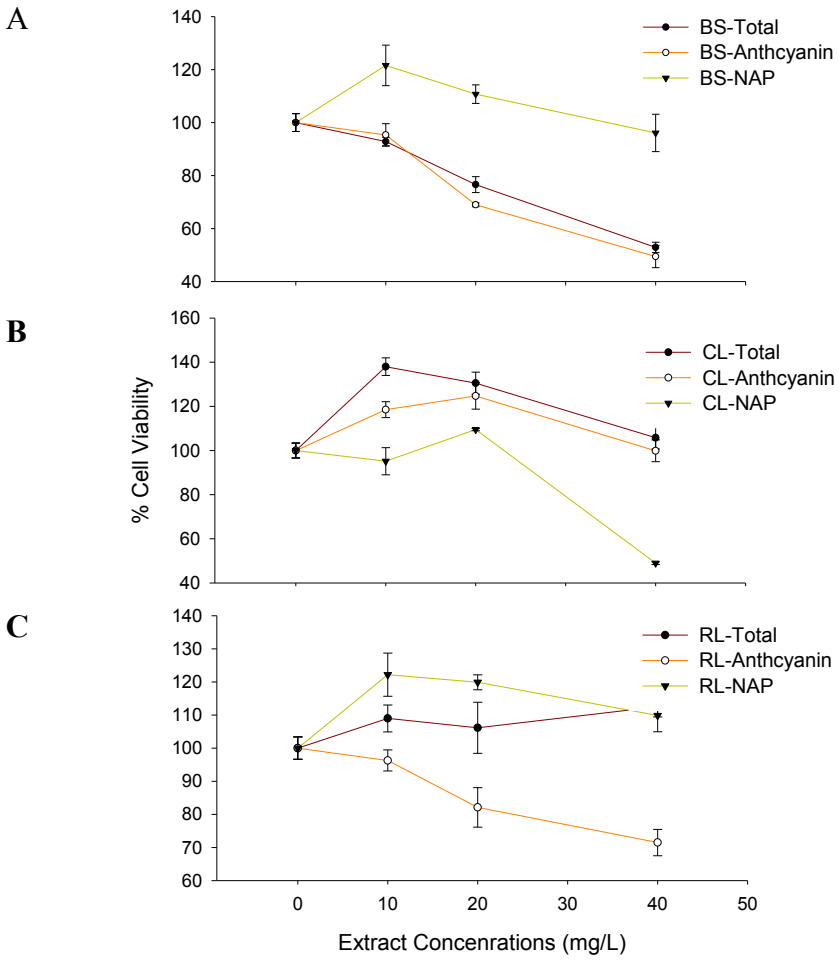


Figure 3.3 Effect on HUVEC cell viability with treatment of fruit extracts on HUVEC. HUVEC cells were seeded (96-well plate) and incubated for 24 hours, followed by treatment with 100µL BS, CL, and RL extracts for 24 hours. Finally, addition of 100µL HUVEC medium and 20µL CellTiter 96® AQueous One Solution Reagent and incubated for 2 hr. Absorbance was read (490nm). Results were expressed in percent viability based on control cells. Graphs representing cell viability of BS plum extracts (A), RL peach extracts (B), and CL peach extracts (C).

The results of the cell-viability assay were used in congruence with the quantification of TSP to correlate the concentration applicable in cell-culture assays to the representative weight of fresh fruit. While this study is aimed at the effect of peach and plum extracts, it is beneficial to know how these values correlate to fresh-fruit

weight (Table 3.3). With that in mind, further studies need to be done to evaluate the bioavailability of these compounds after consumption.

Table 3.3 Concentrations of fruit extract used in the cell culture assays (based on cell-viability assay) for each fraction (total, anthocyanin, and non-anthocyanin polyphenolics) as well as their corresponding value of fresh weight fruit.

Black Splendor Plum		***Rich Lady Peach***		***Crimson Lady Peach***	
concentration used for in-vitro assays *(mg/L)*	***representative grams of fruit*** *(FW)*	***concentration used for in-vitro assays*** *(mg/L)*	***representative grams of fruit*** *(FW)*	***Concentration used for in-vitro assays*** *(mg/L)*	***representative grams of fruit*** *(FW)*
Total		**Total**		**Total**	
10	5.38	10	61.36	15	54.53
5	2.69	5	30.68	7.5	27.27
2.5	1.35	2.5	15.34	3.75	13.63
Anthocyanin		**Anthocyanin**		**Anthocyanin**	
10	7.29	15	83.85	15	73.01
5	3.64	7.5	41.92	7.5	36.50
2.5	1.82	3.75	20.96	3.75	18.25
NAP		**NAP**		**NAP**	
15	26.31	15	128.44	15	157.49
7.5	13.15	7.5	64.22	7.5	78.74
3.75	6.58	3.75	32.11	3.75	39.37

3.3.3. Antioxidant activity of stone fruit extracts

Oxidative stress has been commonly linked to the pathogenesis of vascular lesions (*8, 86*). The antioxidant activity was measured through two assays. First, the Oxygen Radical Absorbance Assay (ORAC) was used to determine the protective effects of the extracts against peroxide radicals. While this is a valid assay, it does not represent potential *in-vitro* anti-oxidant activity. The *in-vitro* antioxidant activity was measured by determining the protective effects of the fruit extracts against hydrogen peroxide-induced radicals in HUVEC cells.

The ORAC assay provided a measurement of the antioxidant capacity of the fruit extracts. This method measures the oxidative degradation of fluorescent molecules after

initiation with the peroxy radical generator, AAPH. Antioxidant capacity is indicated by the ability of the antioxidant to inhibit a decline in fluorescence. BS-total and BS-anthocyanin plum extracts indicated high antioxidant capacities, 12.64 ± 0.95 and 12.12 ± 1.89 μmol TE / g fruit (fresh weight), respectively (Table 3.4). BS-NAP plum fraction results in 4.75 ± 0.15 μmol TE / g fruit (fresh weight), a 62% reduction. This is likely due to the lack of anthocyanins in the BS-NAP plum fraction, suggesting a significant portion of the antioxidant activity comes from the anthocyanin compounds. RL and CL peach extracts elicited a lower ORAC value comparatively to BS plum extracts. The ORAC values of the total, anthocyanin, and NAP fractions for both RL and CL peaches were not significantly different in value, ranging from 0.69 ± 0.05 to 2.6 ± 0.14 μmol TE / g fruit (fresh weight). This would suggest the compounds in the anthocyanin and NAP fractions do not elicit an additive or synergistic effect in the total extract. The high antioxidant capacity of the peach and plum extracts may potentially elicit anti-inflammatory properties as well – due to the correlation of the two events.

Table 3.4 Table represents ORAC value, indicating the antioxidant capacity of fruit extracts against peroxyl-radical generator, AAPH. Fruit extracts were combined with peroxy-radical generator AAPH as well as the fluorescent probe, Fluorescein. Fluorescent activity was measured every 120 seconds for 90 minutes, results expressed in μM Trolox equivalents (TE) per gram of fruit (fresh weight).

Black Splendor Plum	***Rich Lady Peach***	***Crimson Lady Peach***
μmol TE / g fruit (FW)		
Total 12.64 ± 0.95 a	**Total** 1.19 ± 0.03 c	**Total** 2.60 ± 0.14 bc
Anthocyanin 12.12 ± 1.89 a	**Anthocyanin** 1.72 ± 0.22 c	**Anthocyanin** 1.43 ± 0.07 c
NAP 4.75 ± 0.15 b	**NAP** 1.03 ± 0.03 c	**NAP** 0.69 ± 0.05 c

To better estimate the antioxidant activity *in-vitro*, ROS levels in hydrogen peroxide-induced HUVEC cells were quantified with and without treatment of the extracts. HUVEC cells were incubated in 96-well plates, followed by pretreatment with fruit extract for 24hr and then challenged with 100μM H_2O_2 for 2hr. Intracellular ROS was evaluated using H_2DCF-DA staining expressed in relative fluorescence unit (RFU) standardized to the control. The level of intracellular ROS was examined in cells treated with fruit extracts as well as untreated control groups. Exposure of cells to 100μM H_2O_2 resulted in a high ROS level in the untreated HUVEC cells, whereas HUVEC pretreated with varying BS, RL and CL total extract treatments for 24 hours significantly reduced the ROS level by 76, 34, and 39% after exposure to 100μM H_2O_2 compared to their respective control (Figure 3.4).

Pretreatment of HUVEC cells with RL peach extracts resulted in significant reductions in ROS levels (Figure 3.4A). RL-Total extract exhibited protective effects at all treatment doses applied (2.5, 5, and 10 mg/L), with maximum protective effects at 10mg/L eliciting a 34% reduction in ROS levels. RL-Anthocyanins and RL-NAP did not show significant protective action at pre-treatment doses less than 7.5mg/L. However, treatments above 7.5 mg/L of RL-Anthocyanins elicited a significant decrease in ROS levels (25%). RL-NAP showed a maximum protective effect at 15mg/L with a 38% reduction in ROS levels. Each of the RL extracts seems to act protectively, none of them generating a more potent protective effect than the others. Because all fractions elicit the same antioxidant effect, this would suggest the antioxidant activity of the compounds in each fraction is likely not acting synergistically or in an additive nature in the total extract.

Pretreatment of HUVEC cells with CL peach extracts resulted in significant reductions in ROS levels (Figure 3.4B). CL-Total extract elicited a dose dependent reduction in ROS levels. Treatment doses of 2.5, 5, and 10 mg/L resulted in 19, 25, and 39% reduction compared to control cells. CL-Anthocyanin and CL-NAP resulted in significant decreases in ROS levels compared to control, while there was no statistically significant difference between treatment doses. Maximum protective effects of CL-

Anthocyanins and CL-NAP was seen with treatment of 7.5 mg/L (31% reduction in ROS level) and 15mg/L (27% reduction in ROS level), respectively. Similar to RL peach extracts, the CL peach extracts also showed no significant difference in protectiveness between fractions. The same conclusion can be drawn in that, while the peach extracts exhibit significant reduction in ROS levels, the antioxidant activity of the compounds in each fraction is likely not acting synergistically or in an additive nature in the total extract.

Pretreatment of HUVEC cells with BS-Total extracts elicited a much higher protective effect than those of the other fruit extracts, with maximum protectiveness at 10mg/L, reducing ROS levels by 76% after exposure to 100μM H_2O_2 compared to the respective control (Figure 3.4C). Treatment with BS-Anthocyanin and BS-NAP elicited a lesser protective effect, however the reduction from control levels is still statistically significant. There was not a statistically significant difference between treatment doses of BS-Anthocyanin doses and BS-NAP. The maximum protective effects of BS-Anthocyanins and BS-NAP was seen with treatment of 10 mg/L (32% reduction in ROS level) and 15mg/L (38% reduction in ROS level), respectively. Due to the higher protective effects seen from the BS-Total extract, it is possible the compounds in the BS-Anthocyanin fraction and BS-NAP fraction are acting cooperatively, potentially resulting in the strong protective effects seen from the total extract.

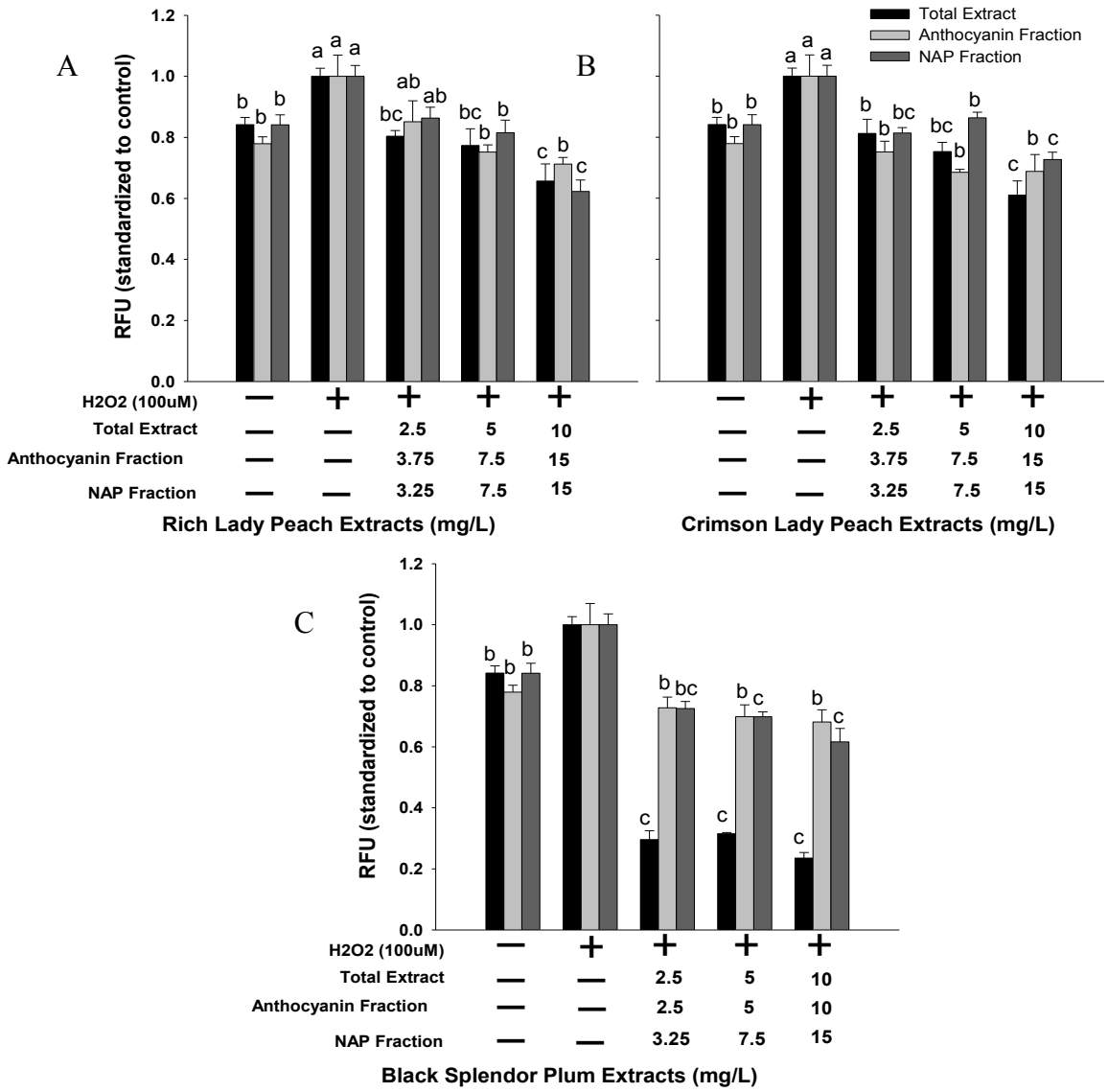


Figure 3.4 Protective effects of fruit extracts against H_2O_2-induced oxidative stress on HUVEC cells. HUVEC were incubated in 96-well plates, followed by pretreatment with fruit extract for 24hr and then challenged with 100μM H_2O_2 for 2hr. Intracellular ROS was evaluated using H_2DCF-DA staining, expressed in relative fluorescence unit (RFU) relative to the positive control. BS plum extracts (A), RL peach extracts (B), and CL peach extracts (C).

3.3.4. Anti-inflammatory activity of stone fruits

Toll-like Receptor-4 (TLR-4), activated by LPS challenge, stimulates signals transmitted through recruitment of more than a dozen different signaling proteins, including IRAK-1 and TRAF-6, forming a signaling cascades leading to NF-κB

activation (*20*). IL-6, IL-8, V-CAM, and COX-2 are up-regulated through the activation of NF-κB and are involved in early vascular lesion development (Figure 2.1). These biomarkers were quantified after LPS induction to determine anti-inflammatory activity of peach and plum extracts.

3.3.4.1. Activation of pro-inflammatory signaling molecules upstream of NF-κB

In efforts to determine whether the LPS-challenge induced inflammation, mRNA expression was measured in LPS-challenged and un-challenged HUVEC cells. HUVEC cells were challenged with 1μg/mL LPS for 3hrs prior to mRNA extraction. Results indicated the 3 hour LPS challenge did not induce pro-inflammatory markers TLR-4, IRAK-1 and TRAF-6 (Figure 3.5). These results were unexpected, as these are central signaling molecules of inflammation. Because no induction was seen in TLR-4, IRAK-1 and TRAF-6, the anti-inflammatory effects of the fruit extracts regarding these pro-inflammatory biomarkers could not be measured. However, a response was seen in mRNA expression of NF-κB (Figure 3.5). A decrease in mRNA expression was seen with treatment of BS, CL, and RL total extracts. Untreated LPS-challenged HUVEC cells elicited an mRNA expression of 1.92. Upon treatment of BS, CL, and RL extracts, the mRNA expression was decreased to 0.84, 0.56, and 1.61 (Figure 3.6A, 3.6B, 3.6C). These results are suggestive of anti-inflammatory activity through disruption of an NF-κB mechanism, seen through a decrease in NF-κB mRNA expression.

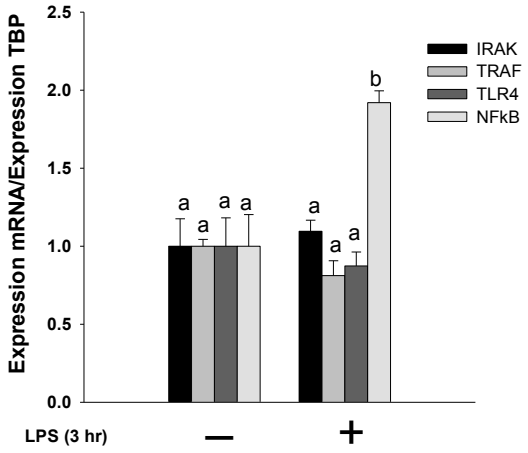


Figure 3.5 mRNA expression of pro-inflammatory biomarkers seen in LPS-stimulated HUVEC cells. Results showed no significant difference in mRNA expression of IRAK-1, TRAF-6, and TLR-4 after LPS challenge, however mRNA expression of NF-κB was increased by nearly 2-fold from control. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$).

The absence of over-expression of mRNA from TLR-4, IRAK-1, and TRAF-6 after LPS induction may suggest NF-κB activity is potentially mediated through a different signaling cascade. Another possibility is the inflammatory affect on TLR-4, IRAK-1 and TRAF-6 has already occurred and returned to basal levels – meriting further studies to determine the optimum cell harvest time period to interpret the inflammatory effect of LPS induction in HUVEC cells.

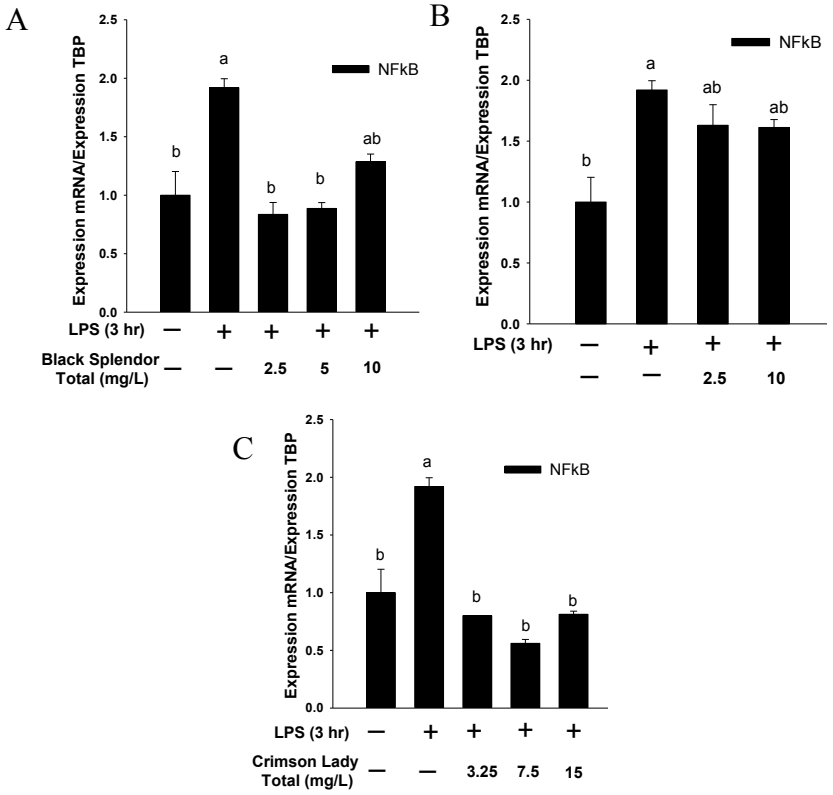


Figure 3.6 A protective effect of fruit extracts on LPS-induced HUVEC cells was seen as a decrease in mRNA expression of pro-inflammatory biomarker, NF-κB. BS Total Extract (A), RL Total Extract (B), and CL Total Extract (C). mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$).

3.3.4.2. NF-κB mediated pro-inflammatory signaling molecules

3.3.4.2.1. Black Splendor plum

In the experiment to determine anti-inflammatory activity of BS extracts in LPS-induced HUVEC cells, pre-treatment doses of BS extracts (total, anthocyanin, and NAP) ranging from 0-15mg/L were applied to HUVEC cells, followed by an LPS-challenge (24hr) to induce an inflammatory response. Cell mRNA expression indicated pretreatment with BS extracts significantly decreased LPS-induced V-CAM, COX-2, IL-6, and IL-8 expression, with little to no significant difference in effectiveness between the ranges of treatment doses used (Figure 3.7). Overall, the results indicate the BS-total extract and BS-anthocyanin fraction elicit a more protective effect through reduction of

mRNA expression of LPS-induced V-CAM and COX-2 (Figure 3.7A, B). There is not a significant difference in the effectiveness between BS-Total extract and the BS-fractions in reduction of LPS-induced IL-6 and IL-8 mRNA expression. The maximum inhibitory effect of LPS-induced V-CAM and COX-2 expression was seen with treatment of BS-total extract (10mg/L) eliciting up to 72% and 77% decreases in mRNA expression from untreated cells, respectively. LPS-induced IL-6 and IL-8 expressions were most inhibited by the BS-anthocyanin fraction, up to 89% and 86% decreases in mRNA expression from untreated cells, respectively. While the maximum inhibitory effects are discussed above, it should be noted that treatment of BS-total extract and BS-fractions elicited a significant decrease in mRNA expression, suggestion anti-inflammatory activity.

These results lead to two suggestions. First, it is possible that the mRNA expression of each pro-inflammatory biomarker responds differently depending on the compounds present in the extract. For example, the anthocyanin fraction had a stronger suppressive effect on mRNA expression of V-CAM and COX-2, while the non-anthocyanin phenolic fraction did not elicit as strong of a response. However, IL-6 and IL-8 were reduced similarly by both fraction treatments. Also, because BS-Total and BS-Anthocyanin treatments elicited similar responses, it may suggest the compounds in each fraction do not show additive or synergistic effects in the total extract – if this was the case we would see a higher protective activity of the total extract compared to the fractions. With these results we can speculate the anthocyanins in BS plum to possibly be the predominantly active class of compounds in regards to anti-inflammatory activity due to the higher overall protective effects seen when compared to BS-NAP (Figure 3.7C).

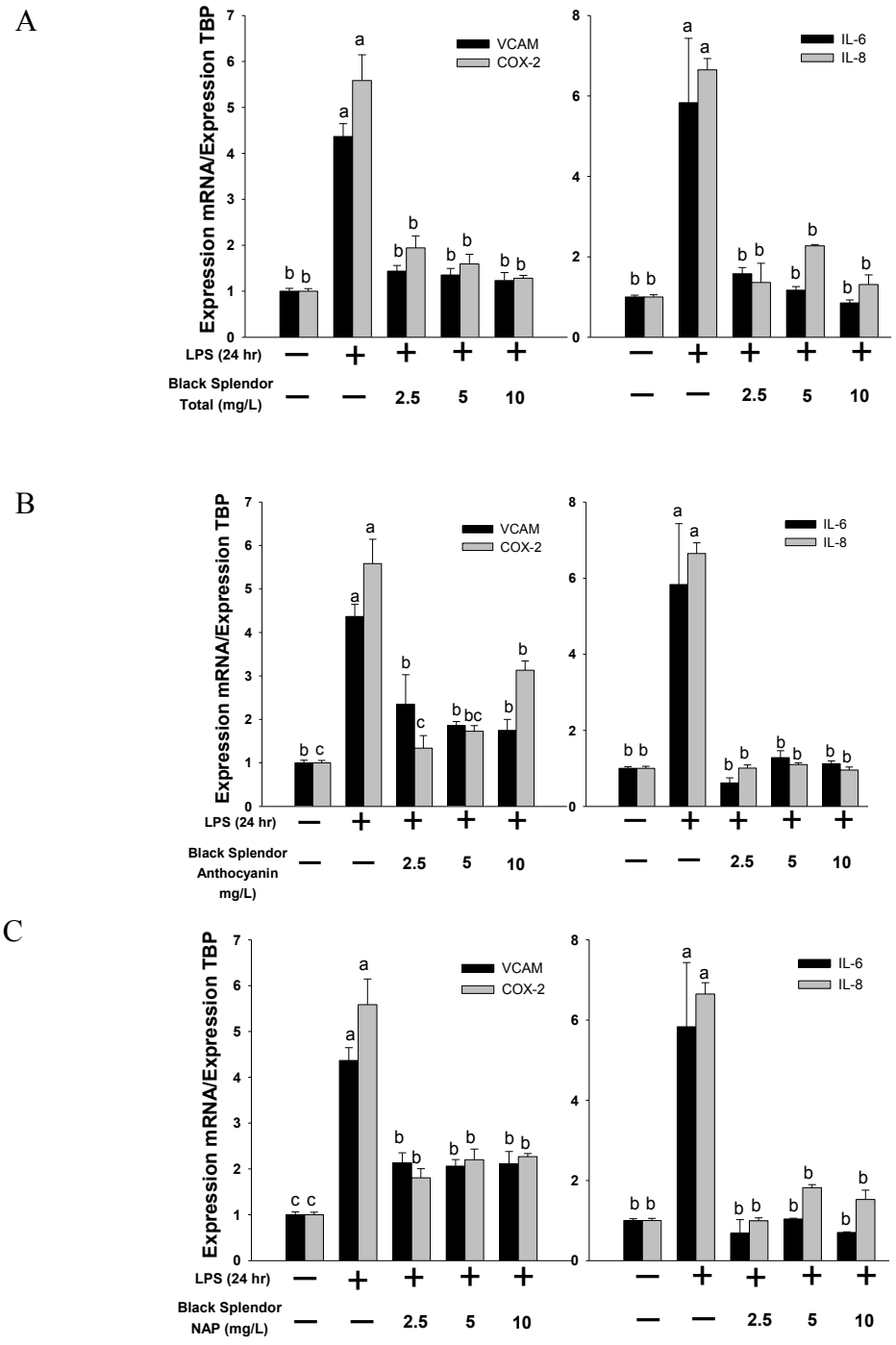


Figure 3.7 The effect of BS total extract (A), BS anthocyanin fraction (B), and BS-NAP fraction (C) on mRNA expression of pro-inflammatory biomarkers, V-CAM, COX-2, IL-6 and IL-8 in LPS-induced HUVEC cells. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$).

3.3.4.2.2. Rich Lady peach

Evaluation of endothelial cell mRNA indicated pretreatment of HUVEC cells with RL extracts (0-15mg/L) significantly decreased mRNA expression of LPS-induced (24hr) V-CAM, COX-2, IL-6, and IL-8 (Figure 3.8). A dose-dependent increase of mRNA expression of V-CAM COX-2, IL-6 and IL-8 is seen with treatment of RL-NAP, likely due to a toxicity effect caused by a combination of the LPS challenge and RL treatment at higher doses. The maximum inhibitory effect of LPS-induced V-CAM and COX-2 mRNA expression was seen with RL-NAP (3.75mg/L) and RL-anthocyanin (15mg/L) generating up to 65% and 73% decreases in mRNA expression from untreated cells, respectively. LPS-induced IL-6 and IL-8 mRNA expression were most inhibited by RL-total (10mg/L) eliciting up to 93% and 91% decreases in mRNA expression from untreated cells, respectively.

These results suggest significant anti-inflammatory activity of RL peach seen through reduction of mRNA expression of pro-inflammatory bio-markers upon treatment of the fruit extract. There does not seem to be a significant difference in the effectiveness between RL-Total extract and the RL-fractions in suppression of LPS-induced mRNA expression of biomarkers. This would suggest the compounds in each fraction (RL-Anthocyanin and RL-NAP) do not show additive or synergistic effects in the RL-Total extract, and that the predominantly active class of compounds in regards to anti-inflammatory activity in RL peach cannot yet be deciphered.

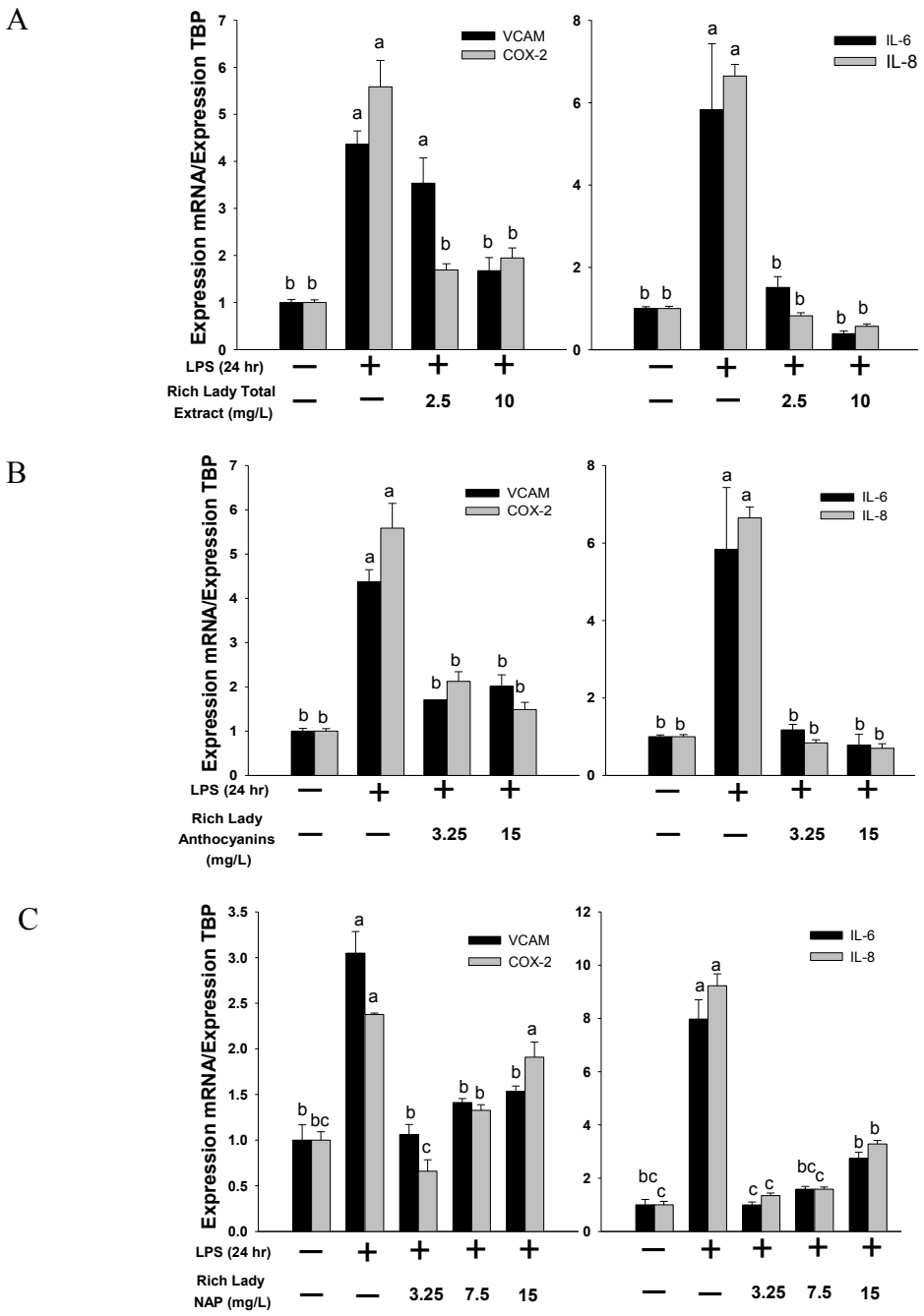


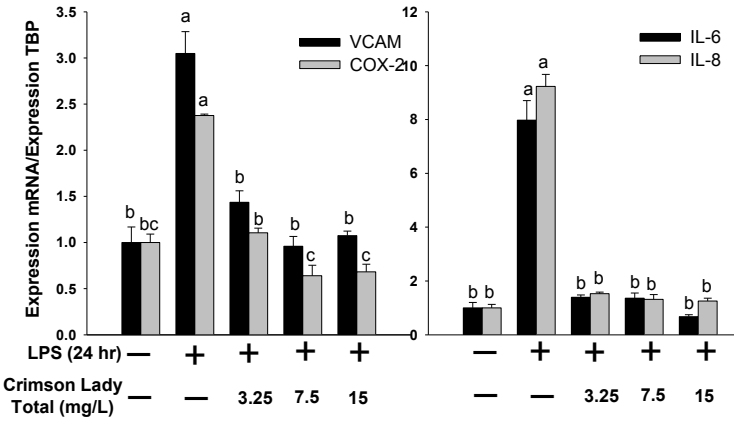
Figure 3.8 The effect of RL total extract (A), RL anthocyanin fraction (B), and RL-NAP fraction (C) on mRNA expression of pro-inflammatory biomarkers, V-CAM, COX-2, IL-6 and IL-8 in LPS-induced HUVEC cells. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$).

3.3.4.2.3 Crimson Lady peach

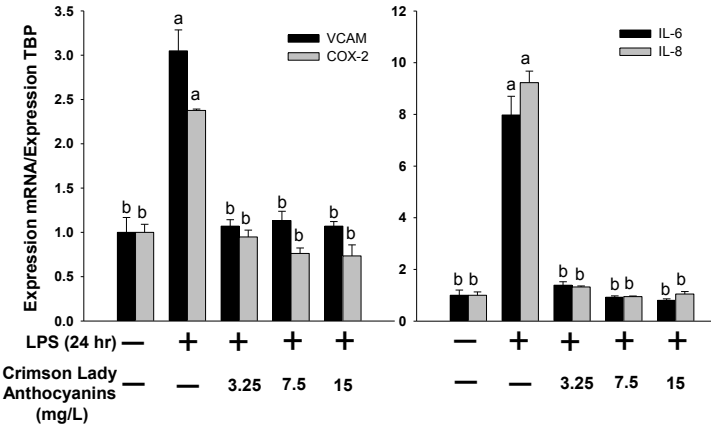
Evaluation of endothelial cell mRNA showed that pretreatment with CL extracts (0-15mg/L) significantly decreased LPS-induced (24hr) mRNA expression of V-CAM, COX-2, IL-6, and IL-8 (Figure 3.9). A dose-dependent increase is seen in mRNA expression of V-CAM and COX-2 with treatment of CL-NAP, likely due to a toxicity effect caused by a combination of the LPS challenge and CL treatment at high doses. The maximum inhibitory effect of LPS-induced mRNA expression of V-CAM, COX-2, and IL-6 was seen with CL-total (7.5, 7.5, and 15mg/L) generating up to a 68, 73, and 92% decrease in mRNA expression from untreated cells, respectively. LPS-induced IL-8 mRNA expression was most inhibited by CL-anthocyanin fraction (7.5mg/L) by up to 90% decreases in mRNA expression from untreated cells.

These results suggest significant anti-inflammatory activity of CL peach seen through reduction of mRNA expression of pro-inflammatory bio-markers upon treatment of the fruit extract. Results indicate CL-Total Extract and CL-Anthocyanin seem to elicit an overall higher anti-inflammatory effect than that of CL-NAP, based on suppression of the selected pro-inflammatory biomarkers upon treatment. This finding suggests the non-anthocyanin polyphenolics in CL peach do not have as high of a protective effect as the anthocyanins, and the main compounds eliciting an inflammatory response in CL-peach extracts may be the anthocyanins.

A



B



C

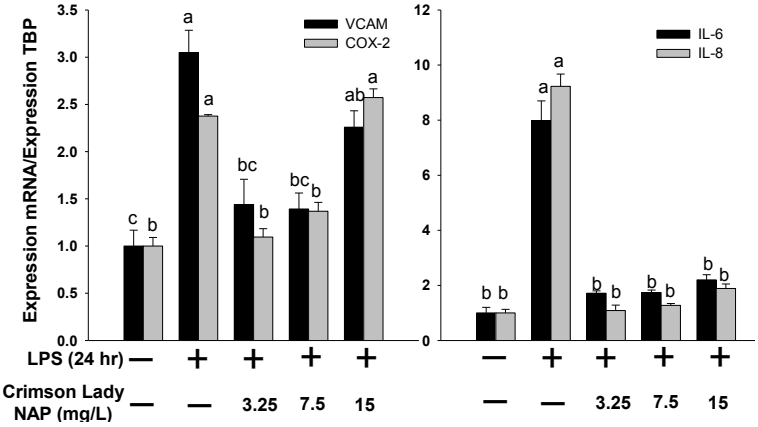


Figure 3.9 The effect of CL total extract (A), CL anthocyanin fraction (B), and CL-NAP fraction (C) on mRNA expression of pro-inflammatory biomarkers, V-CAM, COX-2, IL-6 and IL-8 in LPS-induced HUVEC cells. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$).

3.4. Conclusion

In conclusion, this study suggested that phenolic compounds present in BS, CL, and RL extracts exert significant anti-inflammatory and antioxidant effects on endothelial cells. The secondary plant compound extracts of the peach and plum fruits were proven to have a significant antioxidant activity as well as anti-inflammatory activity. The Black Splendor plum total extract seemed to elicit the highest overall protective effect, however all the tested extracts exhibited significant protectiveness.

Results showed BS-total extract and BS-Anthocyanin fraction to have the highest antioxidant capacity and BS-NAP reported nearly 65% lower activity comparatively. This is likely due to the lack of anthocyanins in the NAP fraction. Both RL and CL peach extracts indicated similar antioxidant capacities which were much lower than that of the BS-plum extracts. This is likely due to the higher reported anthocyanin content of BS plum than that of the RL and CL peaches (*35*). A general trend showed similar ORAC values for the total extract and anthocyanin fractions of the fruits, while the NAP fraction elicited a lower ORAC value. These results suggest a majority of the antioxidant capacity measured by the ORAC assay is coming from the anthocyanins. Protective effects of peach and plum extracts against ROS levels, *in-vitro,* was also measured. These results indicated a very strong protective effect from BS-total extract. The remaining plum and peach extracts elicited a lower, however still significant, protective effect against ROS levels. These results further expose the significant antioxidant effects shown by BS-total extract. Plums have much higher anthocyanin contents than peaches, which is likely the cause of such high antioxidant activity of the BS-total extract.

It is important to note that standardized values were used for *in-vitro* assays allowing the bioefficacy to better be ascribed to the occurrence of specific compounds rather than the higher or lower concentration in the fruit, as a whole. This was appropriate for the scope of this study which was to compare the activities of the phenolic compounds of the fruits for potential use in a health related field, not to determine the effects of fresh fruit consumption by weight. Results suggest BS-total

extract, RL-total extract, and CL-total extract to elicit significant anti-inflammatory activity seen through reduction of mRNA expression of selected bio-markers. In terms of fresh weight consumption, 10mg/L of BS-total, RL-total, and CL-total extracts translates to 5.36, 61.36, and 54.53g fruit (fresh weight).

Overall, the BS, CL, and RL extracts had similar results in suppression of the NF-κB mediated pro-inflammatory biomarkers. Suppression of LPS-induced TLR-4 upon treatment of peach and plum extracts as well as a decrease in activation of downstream signal mediators IRAK-1 and TRAF-6 was expected, however no effect was seen. Significant suppressive effects of inflammatory signaling molecules were seen further down-stream. Suppression of NF-κB as well as the NF-κB mediated pro-inflammatory signaling molecules (IL-6, IL-8, V-CAM-1, and COX-2) was seen with Black Splendor plum, Crimson Lady peach, and Rich Lady peach. These results suggest significant anti-inflammatory activity, however, the mechanism is still undefined.

The potential application for peach and plum fruits would likely target the health related industry. Peach and plum fruits are ideal for use in beverages promoting health and well being as well as marketable to the dietary supplement industry. The results of this study are suggestive of significant benefits from peach and plum fruit phenolics regarding prevention of vascular lesions leading to CVD. The development of dietary supplements as a strategy to prevent or suppress the development of inflammatory lesions in endothelial cells merits further investigation.

4. PHENOLICS FOUND IN EXOTIC FRUITS ELICIT A DECREASE IN OXIDATIVE STRESS AND INFLAMMATION PROVIDING MOTIVATION FOR FURTHER USE WITHIN THE FUNCTIONAL FOOD INDUSTRY

4.1. Introduction

There has recently been a great interest in exotic fruits – more commonly known as "superfruits". The production of exotic fruits is not only important as an economic stimulus to the country of origin, but also provides a source of nutritional dietary requirements for consumers. The cultivation of exotic fruits creates employment opportunities in both the country of fruit origin as well as in upstream import activities. Global production of all fruit types has increased by 19% from 2000-2004, coupled with a 7.5% increase in the export trade value (*87*). In spite of the spike in fruit production in the last five years, there are several challenges to be dealt with in the exotic fruit industry such as production facility constraints, postharvest handling, and marketing opportunities. Identification of the target market and optimum distribution channels are crucial for designing overall export strategies. Exotic fruits are often sold in a variety of processed or semi-processed forms; canned fruit, fruit juices/juice concentrate, fruit pulp or purée, and dehydrated fruit.

The fruits of study for this work are açai, pomegranate, and goji. There has been limited research on these fruits investigating the phenolic content and their potential health benefits, limiting their marketability as functional foods.

Mechanisms of inflammation and oxidative stress are frequently investigated due to their correlation with the Cardiovascular disease (CVD), as discussed in Section 2.1 (*8, 88*). CVD is one of the leading causes of death worldwide (*3*). Vascular disorders, through over-expression of pro-inflammatory adhesion molecules and cytokines (leading to inflammation), are thought to participate in the pathogenesis of atherosclerosis, a common manifestation of CVD (*12*). Overall mortality associated with CVD might be decreased by controlling risk factors such as a high fat diet, high cholesterol, obesity and hypertension which may be modified through an increased intake of fruits and

vegetables (*81, 89*). Plant polyphenolics are increasingly being considered as sources of natural disease preventive compounds on the basis of safety and efficacy assessments through clinical trials. The edible portions of exotic fruits contain phenolics that may protect against inflammatory and oxidative stress leading to vascular lesions.

By analyzing mRNA of LPS-challenged endothelial cells with and without treatment of fruit, the gene expression of pro-inflammatory markers can be quantified to assess the effect on the inflammatory pathway. Lipopolysaccharide (LPS) from bacteria cell walls is an especially potent stimulus for Tumor necrosis factor alpha (TNFα) synthesis (*18*). TNFα is an inflammatory pathway mediating cytokine which binds TNF receptors type II, leading to activation of transcription factor NF-κB. Signals are transmitted through the recruitment of more than a dozen different signaling proteins, which together form signaling cascades (*19*). Activation of NF-κB initiates the transcription of interleukins, cell adhesions molecules, as well as other pro-inflammatory mediators (Figure 2.2). The cytokine, interleukin-6 (IL-6), acts as a pro-inflammatory signaling molecule that is generated in response to NF-κB activation. Cytokines (IL-6 and TNFα) are also initiators of the NF-κB activation pathway. IL-6 further perpetuates inflammation through production of signaling molecules such as IL-8 (a chemokine involved in the up-regulation of inflammatory pathways) (*9*). Thus, creating a positive feedback loop and maintaining a chronic inflammatory environment.

The objective of this screening is to investigate the secondary plant compound composition and understand the anti-inflammatory activity of selected exotic fruits. This information can provide a scientific basis for use in health related industries and increase the potential marketability.

4.2. Materials and methods

4.2.1. *Sourcing*

Fruit was sourced from Stiebs, out of the United States, providing small (100-300 gram) sizes of pomegranate, açai, and goji products. The pomegranate was provided in juice concentrate form (65° Brix 100% Pomegranate Juice Concentrate) from China,

Turkey, India, and the United States. The clarified açai juice from Brazil was processed and clarified at a Stiebs facility (2.5° Brix, 100% pure açai juice). Goji berry puree and concentrate were produced with fresh goji berries grown in China.

Campta, out of Brazil, arranged for 25-35 kg of açai pulp in frozen, 1-kg blocks to be delivered. Additional samples of goji were obtained from Rich Nature (goji concentrate - China) and from Absolute Red (goji puree aseptic - China).

4.2.2. Phenolic extraction for secondary plant compound analysis

For secondary plant compound analysis each fruit was extracted in 100% methanol, filtered, and used directly for spectrophotometric HPLC-PDA and HPLC-MS characterizations. Polyphenolic compounds were analyzed in each fruit, as the fruits came available (due to random arrival of fruits from the various suppliers).

4.2.3. Phenolic extraction for in-vitro analysis

The primary targets for analysis of bioactivity are polyphenolics and related compounds that would have affinity to reversed phase C18. Fruit isolates/concentrates for cell-based assays were made by exhaustive and multiple extractions of each fruit with 100% methanol, filtering, and solvent removal under reduced pressure at 40°C. Isolates were dried using a centrifugal evaporator and re-dissolved in DMSO to form concentrates.

4.2.4. Total soluble phenolics and total anthocyanin content

Folin-Ciocalteu assay was conducted to quantify total reducing equivalents (representative of TSP) in gallic acid equivalents. 100µL sample was added to 1mL 0.25N Folin-Ciocalteu reagent for 3 minutes. Then, 1mL 1N sodium carbonate was added and let stand for 7 minutes. Finally, 7.9mL water was added and let stand for 1 hour. The samples were read at 726nm.

Total anthocyanin contents were determined spectrophotometrically at 510 nm and quantified using mg/kg equivalents of cyanidin-3-glucoside with a molar extinction coefficient of 29,600.

4.2.5. Oxygen radical absorbance capacity assay (ORAC)

ORAC measures the ability of antioxidants to inhibit the decline in fluorescence induced by the peroxy radical generator, AAPH. 50 µL concentrations of fruit extract, 100µL flourescein, and 50µL AAPH solution were quickly injected into a 96 well plate. A standard curve of trolox (0-200µM) was used. The fluorescent activity was measured every 120 seconds for 90 minutes using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC) and expressed as trolox equivalents.

4.2.6. HPLC-PDA and HPLC-ESI-MS

Separations will conducted on a Waters 2695 Alliance system and polyphenolics will be identified and quantified based on spectroscopic characteristics and retention time, as compared to authentic standards. Unidentified flavonoids will be quantified in rutin equivalents, procyanidin-based compounds will be expressed in (+)-catechin equivalents, and phenolic acids in gallic acid equivalents. Mass spectrometric analysis (via chromatographic separations and by direct infusion into the analyzer) will be performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. Electrospray ionization is conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V.

4.2.7. Human umbilical vein endothelial cells (HUVEC)

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in endothelial cell medium (ECM) containing 5% fetal bovine serum, 1% of endothelial cell growth

supplement 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37 °C and 5% CO2, and utilized between passages 4-9.

4.2.8. Cell viability

Cell viability is an important factor when conducting an *in-vitro* assay. The extracts must neither inhibit nor promote normal cell growth. For this experiment, HUVEC cells were treated with the fruit extract, applying a gradient of concentrations from 0-40 mg/L (GAE) and incubated for 24 hours at 37 °C and 5% CO2. The treated cells were visually compared to control (untreated) cells. The highest treatment not affecting cell growth was chosen for use in the remaining *in-vitro* assays. All extracts have been normalized to a standardized phenolic content for *in-vitro* assays. This way the bioefficacy can better be ascribed to the occurrence of specific compounds.

4.2.9. Real-time PCR analysis of messenger-RNAs

HUVEC cells (passage 4-9) were incubated in six-well culture plates at $1x10^5$ cell/ml for 24hr at 37 °C and 5% CO2, allowing for attachment. HUVEC medium was removed and cells washed with PBS, followed by a 30 minute pretreatment of varying BS, CL, and RL concentrations at 37 °C and 5% CO2. Cells were then challenged with 1µg/mL LPS for 24hr exposure periods at 37 °C and 5% CO2.

Total RNA was isolated using the mirVana™ miRNA Isolation Kit Applied Biosystems (AB, Foster City, Ca) following the manufacturer's recommended protocol. Samples were evaluated for quality and quantity using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR according to the manufacturer's protocol. PCR was carried out with the SYBR® Green PCR Master Mix from Applied Biosystems, following manufacturer's protocol, on an ABI Prism 7700 Sequence Detection System (PE Applied). Primers encoding human IL-

6, IL-8, NADPH oxidase and NF-κB were purchased from Integrated DNA Technologies, Inc. (San Diego, CA) and for amplification. Product specificity was examined by dissociation curve analysis.

4.2.10. Superoxide dismutase (SOD) enzyme

HUVEC cells (passage 4-9) were incubated in six-well culture plates at $1x10^5$ cell/ml for 24hr at 37 °C and 5% CO2, allowing for attachment. HUVEC medium was removed and cells washed with PBS, followed by a 24 hour treatment of varying BS, CL, and RL concentrations at 37 °C and 5% CO2. Cells were harvested in the lysis buffer provided by the manufacturer's kit.

Superoxide Dismutase (SOD) is an enzyme that catalyzes the breakdown of superoxide anions to molecular oxygen and hydrogen peroxide. The enzyme is crucial in the cellular antioxidant defense mechanism. SOD enzyme activity was measured following the manufacturers protocol (Superoxide Dismutase Assay Kit). Superoxide radicals are generated by xanthine oxidase and are detected by the formation of formazan die from a tetrazolium salt. Absorbance of the die is read at 450nm, results are given in SOD activity (U/mL).

4.2.11. Statistical analysis

Quantitative data represent mean values with the respective standard error of the mean corresponding to 3 or more replicates. Data was analyzed by one-way analysis of variance (ANOVA) using JMP version 5 (SAS Institute Inc.). Post-hoc Tukey pairwise comparisons were used ($p<0.05$).

4.3. Results and discussion

4.3.1. Antioxidant and anti-inflammatory results of pomegranate extracts

4.3.1.1. Secondary plant compound content and characterization

TSP and total anthocyanins for the pomegranate extracts is seen in Table 4.1. Stiebs pomegranate products exhibited the highest soluble phenolics of all fruits

screened in this study (USA: 1,846 mg/kg, China: 10,125 mg/kg, India: 11,143 mg/kg, Turkey: 12,752mg/kg). These fruits also contained the highest anthocyanin content of all the fruits in the study. The results indicated a significant different in TSP between pomegranates grown in the United States versus those grown in China, India, and Turkey. Pomegranate grown in the United States contained the highest concentration of TSP and total anthocyanins and pomegranates grown in China contained the least, with 45.2% and 92.8% less TSP and anthocyanin content, respectively, than pomegranate grown in United States. The discrepancy in secondary plant compound composition between pomegranate extracts is likely due to the different varietal makeup.

Table 4.1 Summary of total soluble phenolics and total anthocyanins of pomegranate extracts.

Fruit Sample	***Total Soluble Phenolics*** *(mg/kg)*	***Total Anthocyanins*** *(mg/kg)*
Stiebs, Pomegranate Concentrate, USA (~70.1 °Brix)	18,464	1,721.1
Stiebs, Pomegranate Concentrate, China (~69.6 °Brix)	10,125	123.3
Stiebs, Pomegranate Concentrate, India (~70.9 °Brix)	11,143	631.7
Stiebs, Pomegranate Concentrate, Turkey (~71.4 °Brix)	12,752	258.4

The combination of photo diode array (PDA) and mass spectrometry (MS) provided an accurate method for identification and quantification of individual phenolics. The methanol extracts were separated by reverse phase HPLC followed by identification of compounds based on retention time from UV spectra and the fragmentation pattern from compound ionization using the Mass Spectrometry chromatogram.

Identification of phenolic compounds was performed at 360nm, showing mostly ellagitannins. Phenolics were identified by retention time in congruence with the UV-visible spectral data with known standards. HPLC-MS was run on each of the pomegranate fruits to confirm the structure of the compounds on interest (Table 4.2).

The chromatograms for the pomegranate fruits exhibited very similar profiles. The compounds of interest in pomegranates are punicalins, punicalagins, and ellagic acid. The identified compounds of interest were present in differing concentrations between cultivars. The chromatographic profile of Stiebs Pomegranate Concentrate USA (360 nm) indicated the presence of punicalins, punicalagins, and ellagic acid. Similarly, Stiebs Pomegranate Concentrate China (360 nm) portrayed the same elution pattern aside from a wide spread of punicalin peaks. This is true also for Stiebs Pomegranate Concentrate India (360 nm) and Stiebs Pomegranate Concentrate Turkey (360 nm). Both of these also indicated a broad elution time for punicalins (11-117min), however the punicalagin and ellagic acid retention time followed the same trend as the previous pomegranate extracts.

The concentration of the phenolics differed in each pomegranate extract. For the pomegranate extracts grown in China, Turkey, and India the more prevalent phenolic was punicalins followed by punicalagin and much lower concentrations of free ellagic acid. The pomegranate extract from the United States had a significantly higher concentration of punicalagins with much lower presence of punicalins and free ellagic acid. This is possibly due to the region of growth or perhaps post-harvest processing – as the pomegranates from the United States likely have a shorter post-harvest handling time than those from India, Turkey, or China. Pomegranates contain a mixture of punicalins, punicalagin, and free ellagic acids. These phenolic have been heavily researched and are well known for their antioxidant properties (*42, 44, 46, 48, 90*).

Table 4.2 Primary compounds of interest in pomegranate extracts (mg/kg).

	Compound of Interest	***mg/kg***
Stiebs, Pomegranate Concentrate, USA	Total Punicalins	668
	Total Punicalagins	3,152
	Free Ellagic Acid	144
Stiebs, Pomegranate Concentrate, China	Total Punicalins	3,599
	Total Punicalagins	1,222
	Free Ellagic Acid	133
Stiebs, Pomegranate Concentrate, India	Total Punicalins	1,433
	Total Punicalagins	883
	Free Ellagic Acid	128
Stiebs, Pomegranate Concentrate, Turkey	Total Punicalins	2,437
	Total Punicalagins	1,587
	Free Ellagic Acid	353

4.3.1.2. Cell viability

The cell viability assay is used to determine the highest concentration of each extract that will be used on HUVEC cells *in-vitro* that neither inhibit nor promote normal cell growth. The extract-treated cells were visually compared to control (untreated) cells and the highest treatment not affecting cell growth was chosen for use in the remaining *in-vitro* assays (Table 4.3). Results indicated up to 40mg/L of pomegranate extract could be used for *in-vitro* analysis without affecting cell viability and disrupting assay results.

Table 4.3 Effect on HUVEC cell viability with treatment of pomegranate extracts. Table indicates the highest concentration which can be applied to HUVEC cells that do not affect the cell growth.

Fruit Sample	***Extract Concentration used in-vitro*** *(mg/L)*
Stiebs, Pomegranate Concentrate, USA	40
Stiebs, Pomegranate Concentrate, China	40
Stiebs, Pomegranate Concentrate, India	40
Stiebs, Pomegranate Concentrate, Turkey	40

4.3.1.3. Antioxidant effects of pomegranate extracts

The oxygen radical absorbance capacity (ORAC) was measured in efforts to determine the antioxidant capacity of the pomegranate extracts. The results are as expected - fruits with higher anthocyanin content correspond to a higher ORAC value. Extract of pomegranate grown in the USA elicited the highest ORAC value (204 μmol TE/g) while the extracts of pomegranates grown in China, India and Turkey elected ORAC values from 98 – 121 μmol TE/g. The disagreement in ORAC value of extracts of the same fruit is likely due to the difference secondary plant compound composition as the pomegranates are of different varieties. The high ORAC value of pomegranates grown in the USA could be due to the higher punicalagin concentration, as compared to the other varieties. Pomegranates grown in China, India and Turkey contain a much higher concentration of punicalins where pomegranates grown in the USA contain a higher concentration of punicalagins. Pomegranates grown in the USA indicated punicalagin content 50% higher than those in Turkey, 61% higher than those grown in China, and 72% higher than those grown in India (Table 4.4).

Table 4.4 Table represents ORAC value, indicating the antioxidant capacity of fruit extracts against peroxyl-radical generator, AAPH. Fruit extracts were combined with peroxy-radical generator AAPH as well as the fluorescent probe, Fluorescein. Fluorescent activity was measured every 120 seconds for 90 minutes, results expressed in μM Trolox equivalents (TE) per gram of fruit (fresh weight).

Fruit Sample	***ORAC*** *(μmol TE/g)*
Stiebs, Pomegranate Concentrate, USA	204.1
Stiebs, Pomegranate Concentrate, China	98.3
Stiebs, Pomegranate Concentrate, India	115.5
Stiebs, Pomegranate Concentrate, Turkey	121.3

To better estimate the antioxidant activity *in-vitro*, mRNA expression of the pro-oxidant enzyme, NADPH oxidase, was quantified and activity of the antioxidant enzyme, superoxide dismutase (SOD), was measured. An LPS challenge leads to a

NADPH-dependent oxidative burst. Cytosolic enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase transfer electrons from NADPH across cell membranes and are a major source of cytoplasmic ROS, specifically superoxide radicals ($O_2^{\cdot-}$). Studies reported increased cellular NADPH oxidase levels to be associated with increased superoxide levels – suggesting a central role in the inflammatory response. Super oxide dismutase (SOD) is a ubiquitous enzyme which acts as a superoxide radical inhibitor (*20*). Superoxide radicals, when present in excess, are implicated in the development of vascular diseases such as atherosclerosis through activation of the redox-sensitive transcription factor, NF-κB, however the mechanisms are incompletely defined. Antioxidant compounds reported to reduce *in-vitro* ROS levels are also shown to attenuate TNFα mediated activation of the NK-κB inflammatory signaling cascade (*20*).

Extracts of pomegranates grown in Turkey (Figure 4.1B) and China (Figure 4.1C) reduced mRNA expression of LPS-induced NADPH oxidase by 96% and 94% compared to untreated cells, thereby suggesting a decrease in production of superoxide radicals. SOD activity was decreased by 68% and 77% with treatment of pomegranates grown in Turkey and China. A decrease in the activity of the antioxidant enzyme could be due to the antioxidant activity provided by the pomegranate extract, leading to an innate decrease in SOD activity. Treatment of HUVEC with the pomegranate extract from US resulted in a 61% decrease in mRNA expression of NADPH oxidase (Figure 4.1A), however this effect was seen only at low concentrations (5mg/L). The same trend was seen with SOD enzyme activity. The dose dependent increase of NADPH oxidase mRNA expression and antioxidant enzyme SOD activity upen treatment of HUVEC with pomegranate-USA suggests antioxidant activity at low concentrations followed by an induction of cell-stress at concentrations of 10mg/L or higher. A potential explanation could be that pomegranates grown in the USA contain a larger concentration of punicalagins, a high molecular weight compound, than pomegranates grown in Turkey, China and India. The variety from the US also contains much higher anthocyanin content. It is possible the high punicalagin or anthocyanin content may be detrimental to

the cells, eliciting cell-stress. Extracts of pomegranates grown in Turkey and China suggest a decrease in reactive oxygen species production *in-vitro*. These varieties contain lower punicalagin contents and higher punicalin contents, which may be the key to their antioxidant activity seen through suppression of NADPH oxidase and SOD enzymes.

The extract of pomegranate grown in India did not significantly affect the mRNA expression of LPS-induced NADPH oxidase, suggesting continuation of superoxide radical generation (Figure 4.1D). SOD activity was increased nearly 76% from control. This effect most likely occurred to neutralize radical activity generated by NADPH oxidase. Thus, extracts of pomegranate grown in India are suspected to have little to no antioxidant activity, based on mRNA expression of NADPH oxidase and SOD activity. The pomegranate variety from India contains a lower level of punicalins than the varieties from Turkey and China, however higher than the variety grown in the US. Given the results we have seen through comparison of these varieties, it may be hypothesized the antioxidant activity is coming from punicalin content, and not from the punicalagin content.

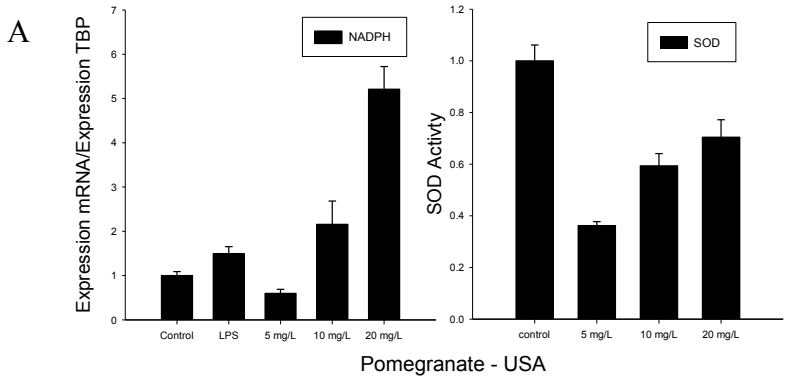


Figure 4.1 mRNA expression of pro-oxidant marker NADPH oxidase in HUVEC cells with 24hr LPS challenge (1μg/mL) and 30min pretreatment of fruit extract. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM (n ≥ 3). Activity of anti-oxidant enzyme Super Oxide Dismutase (SOD) in HUVEC cells with 24hr treatment of fruit extract [A- USA; B-Turkey; C-China; D-India].

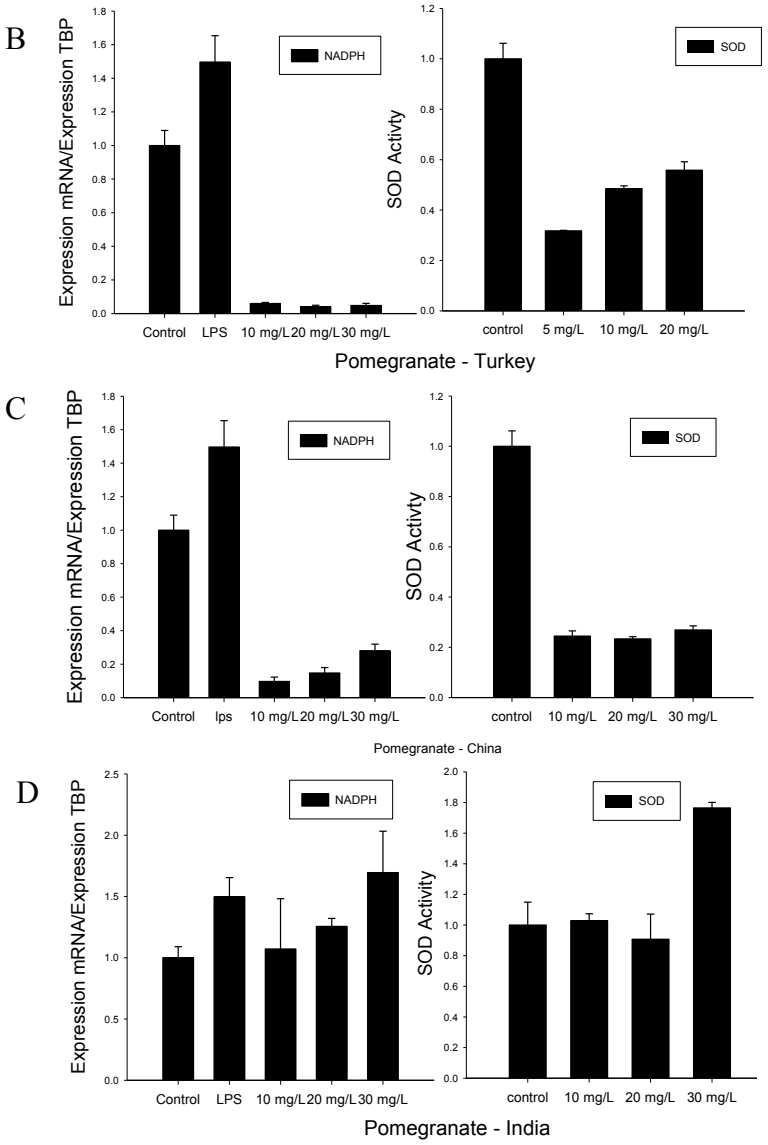


Figure 4.1 Continued.

4.3.1.4 Anti-inflammatory effects of pomegranate extracts

Extracts of pomegranate grown in USA, Turkey and China suppressed the LPS-induced increase of pro-inflammatory biomarkers NF-κB, TNFα, IL-8 and IL-6. Treatment doses ranged from 10-30mg/L and the maximum inhibitory effect of extract of pomegranate grown in US was observed at 5mg/L (figure not shown). Higher

concentrations of pomegranate from USA were no longer protective. The pre-treatment of pomegranates from USA elicited at decrease in pro-inflammatory biomarker expression (NF-κB, TNFα and IL-8) in the range of 60-85% (Table 4.5A,B). mRNA expression of IL-6 was decreased by only 11% (Table 4.5B).

Extracts of pomegranate grown in Turkey and China suggested an anti-inflammatory effect, however the treatments did not respond in a dose-dependent manner. Treatment doses for both extracts were 10, 20, and 30 mg/L. Pomegranate from Turkey indicated anti-inflammatory activity with a 24 and 27% decrease in mRNA expression of LPS-induced IL-6 and IL-8 (Table 4.5B). The extract had a stronger suppressive effect on LPS-induced NF-κB and TNFα, eliciting a 39 and 46% decrease in mRNA expression from untreated cells (Table 4.5A). Similar results were seen with pomegranate from China, in which the strongest suppressive effect was seen as a 76% decrease in mRNA expression of TNFα (Table 4.5A). These results suggest strong anti-inflammatory activity of these extracts targeting the TNF inflammatory pathway.

Extract of pomegranate grown in India elicited an even larger suppression of the LPS-induced pro-inflammatory biomarkers with treatments up to 30 mg/L. Up to 81%, 93%, 94% and 87% decreases in mRNA expression of LPS-induced NF-κB, TNFα, IL-8 and IL-6 was seen compared to untreated cells with treatment of pomegranate from India (Table 4.5A,B). All concentrations of the pomegranate extract provided a protective effect. This suggests strong anti-inflammatory activity targeting the TNF inflammatory pathway.

Table 4.5 Table indicates the mRNA expression of pro-inflammatory markers (A) NF-κB and TNFα and (B) Il-6 and IL-8 in HUVEC after a 24hr LPS-challenge (1μg/mL) in untreated HUVEC (control) and a 30min pretreatment with pomegranate extract (treated cells). The treatment (5-30mg/L) corresponds to the pomegranate extract of the specified growing region listed in the first column. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM (n ≥ 3).

A

	NF-kB		**TNFa**	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Turkey	2.00 ± 0.13	1.22 ± 0.07	69.32 ± 13.18	37.75 ± 8.69
China	1.93 ± 0.14	1.16 ± 0.07	17.70 ± 1.46	4.22 ± 0.06
USA	2.00 ± 0.13	0.75 ± 0.05	44.64 ±5.15	10.76 ± 1.33
India	2.00 ± 0.13	0.38 ± 0.06	17.70 ± 1.46	1.31 ± 0.33

B

	IL6		***IL8***	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Turkey	4.7 ± 0.38	3.56 ± 0.41	34.20 ± 1.83	24.96 ± 0.97
China	4.45 ± 0.07	2.59 ± 0.07	52.00 ± 0.03	27.61 ± 1.19
USA	5.97 ± 0.45	5.28 ± 0.44	34.2 ± 1.83	5.0 ± 0.16
India	4.7 ± 0.38	0.61 ± 0.05	34.2 ± 1.83	1.96 ± 0.43

The prominent, and often regarded therapeutically relevant, compounds in pomegranates are ellagic acid, ellagitannins (punicalins and punicalagins), and anthocyanins. These compounds are proven to be synergistic and elicit many health benefits such as antioxidant, anti-inflammatory, and anti-tumoral effects (*38, 44-46, 54*). Pomegranate extract from USA contains a considerably higher TSP and anthocyanin content (18 g/kg and 1,721 mg/kg, respectively) than the TSP and anthocyanin content from cultivars from China, India, and Turkey (10-12 g/kg and 123-631 mg/kg, respectively). HPLC analysis showed a large variation in concentration of ellagic acid, punicalagins, and punicalins. Pomegranate extract from India contains much higher anthocyanin content than those of Turkey and China, however, did not target the anti-oxidant markers. This suggests the anthocyanins, which are well known for their antioxidant activity, may have less of a contribution to the antioxidant and anti-

inflammatory mechanisms for this particular extract. These results indicate the variation seen in anti-inflammatory and antioxidant activity may be due to range of concentrations of bioactive compounds between pomegranate extracts.

4.3.2. Antioxidant and anti-inflammatory results of açai extracts

4.3.2.1. Secondary plant compound content and characterization

Açai berries are known for their rich secondary plant compound composition and high anthocyanin content (Table 4.6). This data showed the TSP of Campta açai puree is nearly 2.9 times larger than the TSP content of Stiebs clarified açai juice. The total anthocyanin content of Campta açai puree is 5.3 times the total anthocyanin content of Stiebs clarified açai juice. It has been found that the clarification procedure may have an initial detrimental effect on phenolics because polymeric anthocyanins can be removed from the juice (*58*). This reasoning could be the explanation for the drastic difference in TSP and total anthocyanin content between the two açai extracts.

Table 4.6 Summary of total soluble phenolics and total anthocyanins of açai extracts.

Fruit Sample	***Total Soluble Phenolics*** *(mg/kg)*	***Total Anthocyanins*** *(mg/kg)*
Stiebs Clarified Açai Juice	1,896	245
Campta Açai puree	5,498	1,294

Identification of phenolic compounds present in Stiebs Clarified Açai Juice (100% Juice) and Campta Açai Pulp was analyzed at 280nm, indicating mostly phenolic acids, 360nm showing mostly flavonoid-glycosides, and 510nm showing anthocyanins. Phenolics were identified by retention time in congruence with UV-visible spectral data with known standards. HPLC-MS was run on both Açai extracts to verify the structures of the compounds of interest (Table 4.7).

Stiebs Clarified Açai Juice (100% Juice) indicated a low presence of phenolics based on the HPLC-MS data. The compounds identified by their HPLC-MS data were

flavonoids. These included apigenin di-glycoside, taxifolin deoxyhexose, orientin, isoorientin derivative, luteolin di-glycoside, vitexin, isovitexin derivative, scoparin, and a quercetin glycoside. There are a large amount of unknown compounds showing UV-spectral data in the 280nm range, suggesting phenolic acids. The HPLC-MS chromatograms of the two açai extracts showed the presence of similar compounds. Campta Açai Puree showed the aforementioned flavonoids in much higher concentrations. Campta Açai Puree also indicated the presence of the anthocyanin, cyanidin glycoside.

The data collected in this study is congruent with that of previous studies. One study claimed Açai pulp contained anthocyanins, the two major compounds being cyanidin 3-glucoside and cyanidin 3-rutinoside (*58, 60*). Previously, four flavon-3-ol derivatives, five phenolic acids, (-)-epicatechin, and (+)-catechin were also identified (*58, 60-62*). These compounds have been the subject of many health-related studies due to their high antioxidant properties and increasing demand in the food industry.

Table 4.7 Primary compounds of interest in açai (mg/kg).

	Compound of Interest	***mg/kg***
Stiebs Clarified Açai Juice	Total by HPLC	340.4
Campta Açai Pulp	Total by HPLC	1,313

4.3.2.2. Cell viability

The cell viability assay is used to determine the highest concentration of each extract that will be used on HUVEC cells *in-vitro* that neither inhibit nor promote normal cell growth. The extract-treated cells were visually compared to control (untreated) cells and the highest treatment not affecting cell growth was chosen for use in the remaining *in-vitro* assays (Table 4.8).

Each extract must be tested individually, as they contain a plethora of secondary plant compounds that may have an inhibitory effect of cell growth. For this study, anti-inflammatory activity was measured by quantifying expression of pro-inflammatory

biomarkers in LPS-challenged HUVEC cells. The difference in expression between cells treated with fruit extract and cells without treatment was measured to determine the potential anti-inflammatory effect of the fruit extract. For this reason, it is important to ensure fruit extracts treatments will not interfere with cell growth or cause cell death, as that will hinder the results.

Table 4.8 Effect on HUVEC cell viability with treatment of açai extracts. Table indicates the highest concentration which can be applied to HUVEC cells that do not affect the cell growth.

Fruit Sample	***Extract Concentration used in-vitro (mg/L)***
Stiebs, Clarified Açai Juice	20
Campta, Açai puree	20

4.3.2.3. Antioxidant effects of açai extracts

The oxygen radical absorbance capacity (ORAC) was measured in efforts to determine the antioxidant capacity of the açai extracts (Table 4.9). The results are as expected – Campta açai puree elicited a higher ORAC value than Stiebs clarified açai juice, likely due to the higher anthocyanin content of the puree. As discussed previously, it has been suggested the clarification process removes polymeric anthocyanins, thereby decreasing the TSP content and their potential antioxidant activity.

Table 4.9 Table represents ORAC value, indicating the antioxidant capacity of fruit extracts against peroxyl-radical generator, AAPH. Fruit extracts were combined with peroxy-radical generator AAPH as well as the fluorescent probe, Fluorescein. Fluorescent activity was measured every 120 seconds for 90 minutes, results expressed in μM Trolox equivalents (TE) per gram of fruit (fresh weight).

Fruit Sample	***ORAC*** *(μmol TE/g)*
Stiebs, Clarified Açai Juice	31.0
Campta, Açai puree	142.4

To better estimate the antioxidant activity *in-vitro*, mRNA expression of the pro-oxidant enzyme, NADPH oxidase, was quantified and activity of the antioxidant enzyme, superoxide dismutase (SOD), was measured (Figure 4.2). mRNA expression of LPS-induced NADPH oxidase was reduced by Stiebs and Campta açai products by up to 95% and 96% compared to untreated cells, respectively. Suppression of NADPH oxidase activity would suggest a decrease in production of superoxide radicals. The maximum protective effect of Stiebs and Campta açai products was seen at 10mg/L and 5mg/L, respectively. Activity of the antioxidant enzyme, SOD, was also decreased by up to 61% and 55% upon treatment of Stiebs and Campta açai products, respectively. While SOD activity is decreased at low treatment doses, both Stiebs and Campta açai products do not affect SOD activity at higher concentrations. The decrease in SOD activity could possibly be a cellular homeostatic response due to the antioxidant capacity provided by the açai products. Stiebs and Campta açai products suggest significant suppression of reactive oxygen species production *in-vitro* through suppression of enzyme activity of NADPH oxidase and SOD.

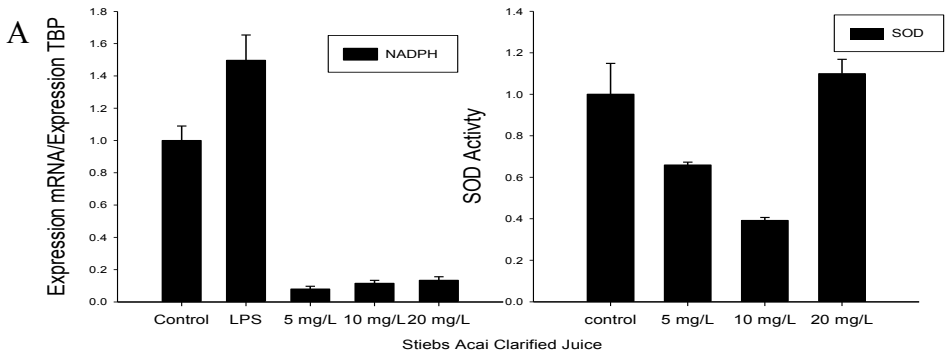


Figure 4.2 mRNA expression of pro-oxidant marker NADPH oxidase in HUVEC cells with 24hr LPS challenge (1µg/mL) and 30min pretreatment of açai extract. mRNA was analyzed by RT-PCR as ratio to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$). Activity of anti-oxidant enzyme Super Oxide Dismutase (SOD) in HUVEC cells with 24hr treatment of *açai* extract [A- Stiebs clarified pomegranate juice; B-Campta *açai* puree].

B

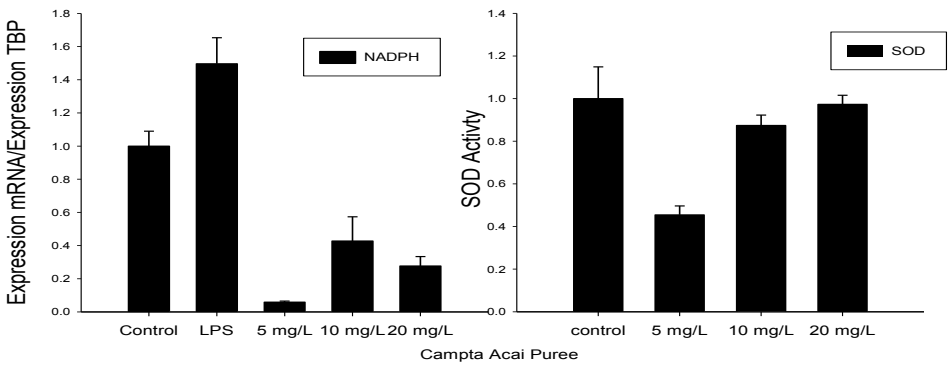


Figure 4.2 Continued.

4.3.2.4. Anti-inflammatory effects of açai extracts

Stiebs clarified açai juice and Campta açai puree suppressed the LPS-induced increase of mRNA expression of pro-inflammatory biomarkers NF-κB, TNFα, IL-6 and IL-8 (Table 4.10). Treatment doses ranged from 5-20mg/L, eliciting a maximum inhibitory effect on the biomarker, TNFα. Campta and Stiebs açai products decreased mRNA expression of TNFα by 90% and 98%, respectively, compared to untreated cells. Both açai products elicited an inhibitory effect on the remaining pro-inflammatory biomarkers as well, however at lower levels. Campta açai puree decreased mRNA expression of NF-κB, IL-6 and IL-8 by 46%, 56% and 77%, respectively, relative to untreated cells. Stiebs clarified açai juice elicited similar protective effects. A decrease in mRNA expression of NF-κB, IL-6 and IL-8 by up to 59%, 76% and 70%, respectively, relative to untreated cells. These results suggest strong anti-inflammatory activity through targeting suppression of pro-inflammatory biomarkers along the TNF inflammatory pathway.

Table 4.10 Table indicates the mRNA expression of pro-inflammatory markers (A) NF-κB and TNFα and (B) Il-6 and IL-8 in HUVEC after a 24hr LPS-challenge (1μg/mL) in untreated HUVEC (control) and a 30min pretreatment with açai extract (treated cells). The treatment (10-30mg/L) corresponds to the extract listed in the first column. mRNA was analyzed by RT-PCR as ratio to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$) .

A

	NF-kB		TNFa	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Campta açai puree	2.00 ± 0.13	1.08 ± 0.19	17.70 ± 1.46	1.71 ± 0.35
Stiebs clarified juice	2.00 ± 0.13	0.82 ± 0.06	17.70 ± 1.46	0.44 ± 0.04

B

	IL-6		IL-8	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Campta açai puree	5.11 ± 0.5	2.25 ± 0.37	34.2 ± 1.83	7.86 ± 0.56
Stiebs clarified juice	5.11 ± 0.5	1.21 ± 0.11	34.2 ± 1.83	10.41 ± 0.86

The two açai products have similar secondary plant compound compositions, however, results from mass spectrometry suggest a significant difference in concentration of these compounds. Campta açai puree contained higher TSP (5,498 mg/kg) and anthocyanin content (1,294 mg/kg) than that of Stiebs clarified açai juice (1,896 mg/kg TSP and 31 mg/kg anthocyanin content). The ORAC value (142.4 TE/g) is nearly 4.5 fold more than that of the Stiebs açai clarified juice. However, Stiebs clarified açai juice showed slightly higher anti-inflammatory and antioxidant activity than Campta Açai puree. This could be due to a multitude of reasons, perhaps due to the better availability of compounds in clarified juice as opposed to the puree. The matrix of chemical composition within an extract can largely affect the availability and activity of the extract, which should be considered during evaluation of secondary plant compound composition. As portrayed in these results, the analysis of secondary plant compounds is an important step in investigating the bioactivity of a food matrix, however not necessarily fully representative of the activities of the secondary plant compounds *in-vitro*.

4.3.3. Antioxidant and anti-inflammatory results of goji berry extracts

4.3.3.1. Secondary plant compound content and characterization

The four goji extracts studied in this screening were obtained from 3 separate suppliers. Stiebs provided a goji puree and a goji puree concentrate. The goji puree has a TSP content of 3,228 mg/kg while the puree concentrate contains 5,482 mg/kg TSP (Table 4.11). Absolute Red provided a goji puree with a TSP content of 3,120 mg/kg, similar in concentration to the Stiebs goji puree. Finally, Rich Nature provided a goji concentrate with had the lowest TSP content, at 1,824 mg/kg. The goji concentrate provided by Rich Nature would be expected to have had a higher TSP, however the results prove otherwise. This may be due to processing techniques or varietal differences.

Table 4.11 Summary of total soluble phenolics of goji extracts.

Fruit Sample	***Total Soluble Phenolics*** *(mg/kg)*
Stiebs Goji Puree Concentrate (42.5 °Brix) China	5,482
Stiebs Goji Puree (17.5 °Brix) China	3,228
Absolute Red Goji (Aseptic, single strength)	3,120
Rich Nature Goji Concentrate	1,824

Considerable research has been done on the goji berry, however most of this is focused on the unique secondary plant compound compounds, such as protein-polysaccharides, and leaving the common phenolics derived from plants, such as phenolic acids and flavonoids, less characterized. The combination of photo diode array (PDA) and mass spectrometry (MS) provided an accurate method for identification and quantification of individual phenolics.

Identification of phenolic compounds present in Stiebs goji puree concentrate, Absolute Red goji puree and Rich Nature goji concentrate was analyzed at 280nm, indicating mostly phenolic acids; and 360nm indicating mostly flavonoid glycosides.

Phenolics were identified by retention time in congruence with UV-visible spectral data with known standards. HPLC-MS was used to verify structures of the compounds of interest (Table 4.12).

The goji berry extracts had similar phenolic profiles. Flavonoid glycosides, di-caffeoylquinic acids, and unknown poly-glycosylated compounds were the major phenolics identified. The polysaccharide-protein complexes found in goji berries may be esterified to phenolic compounds leading to poly-glycosylated compounds. This study seems to be the first discovery of di-caffeoylquinic acid in goji berries, as the ionization pattern matches that of di-caffeoylquinic acid found in yerba mate (*91*). Stiebs goji puree concentrate is rich in di-caffeoylquinic acid (307.44 mg/kg), while the Absolute Red and Rich Nature goji extracts have nearly 84% less. This abundance of either of these compounds in Stiebs goji puree concentrate could be the cause of the lower bioactivity, as discussed later. Considerable research on goji berries has focused on the polysaccharide-protein complexes reported to improve immune defenses as well as anti-cancer activity (*71*). The literature on polyphenolics in goji is growing rapidly and reporting significant health benefits such as anti-cancer and antioxidant activities (*68, 71*).

Table 4.12 Primary compounds of interest in goji berry (mg/kg).

	Compound of Interest	***mg/kg***
Stiebs Goji Puree Concentrate	Tri-glycosyated compounds	205.77
	Di-caffeoylquinic acids	307.44
	Tetra-glycosyated compounds	594.08
	Flavonoid (di-glycoside)	205.68
	Flavonoid (tri-glycoside)	46.96
Absolute Red Goji Puree	Tri-glycosyated compounds	75.9
	Di-caffeoylquinic acids	47.14
	Tetra-glycosyated compounds	73.68
	Poly-glycosylated compound	184.83
	Flavonoid (di-glycoside)	26.87
	Flavonoid (tri-glycoside)	16.14
Rich Nature Goji Concentrate	Di-caffeoylquinic acids	51.85
	Poly-glycosylated compound	332.59
	Flavonoid (di-glycoside)	72.94

4.3.3.2. Cell viability

The cell viability assay is used to determine the highest concentration of each extract that will be used on HUVEC cells *in-vitro* that neither inhibit nor promote normal cell growth. The extract-treated cells were visually compared to control (untreated) cells and the highest treatment not affecting cell growth was chosen for use in the remaining *in-vitro* assays (Table 4.13).

Each extract must be tested individually, as they contain a plethora of secondary plant compounds that may have an inhibitory effect of cell growth. For this study, anti-inflammatory activity was measured by quantifying expression of pro-inflammatory biomarkers in LPS-challenged HUVEC cells. The difference in expression between cells treated with fruit extract and cells without treatment was measured to determine the potential anti-inflammatory effect of the fruit extract. For this reason, it is important to ensure fruit extracts treatments will not interfere with cell growth or cause cell death, as that will hinder the results.

Table 4.13 Effect on HUVEC cell viability with treatment of goji extracts. Table indicates the highest concentration which can be applied to HUVEC cells that do not affect the cell growth.

Fruit Sample	***Extract Concentration used in-vitro (mg/L)***
Stiebs, Goji Puree Concentrate, China (42.5 °Brix)	40
Stiebs, Goji Puree, China (17.5 °Brix)	40
Absolute Red, Goji (Aseptic, single strength)	40
Rich Nature, Goji Concentrate	40

4.3.3.3. Antioxidant effects of goji berry extracts

In general, fruits which contain a higher TSP content tend to have a higher antioxidant capacity – suggesting the phenolic compounds present in these fruits to have significant antioxidant activity. In efforts to quantify the antioxidant activity of the goji extracts, the Oxygen Radical Absorbance Capacity (ORAC) value was measured. Stiebs goji puree concentrated elicited the highest ORAC value, which is likely due to the rich phenolic composition, as compared to the other goji extracts (Table 4.14). Results indicate the extent of the ORAC value is consistent with the corresponding TSP content.

Table 4.14 Table represents ORAC value, indicating the antioxidant capacity of fruit extracts against peroxyl-radical generator, AAPH. Fruit extracts were combined with peroxy-radical generator AAPH as well as the fluorescent probe, Fluorescein. Fluorescent activity was measured every 120 seconds for 90 minutes, results expressed in μM Trolox equivalents (TE) per gram of fruit (fresh weight).

Fruit Sample	***ORAC*** *(μmol TE/g)*
Stiebs Goji Puree Concentrate (42.5 °Brix) China	91.9
Stiebs Goji Puree (17.5 °Brix) China	59.2
Absolute Red Goji (Aseptic, single strength)	69.5
Rich Nature Goji Concentrate	82.1

To better estimate the antioxidant activity *in-vitro*, mRNA expression of the pro-oxidant enzyme, NADPH oxidase, was quantified and activity of the antioxidant enzyme, superoxide dismutase (SOD), was measured (Figure 4.3). Treatment of LPS-induced HUVEC with Stiebs goji puree concentrate significantly reduced mRNA expression of NADPH oxidase (91% reduction compared to untreated cells), thereby decreasing the production of superoxide radicals. Treatment with Rich Nature goji concentrate, Stiebs goji puree, and Absolute Red goji all suggested similar antioxidant activity, eliciting a decrease in mRNA expression of NADPH oxidase by up to 83%, 87% and 91% compared to untreated cells. Treatment with LPS-induced HUVEC with Stiebs goji puree concentrate also suppressed SOD enzyme activity up to 73% compared to untreated cells. A decrease in the activity of the antioxidant enzyme, SOD, could be a cellular homeostatic response due to the antioxidant capacity provided by the goji extract. Again, similar antioxidant results were seen with Rich Nature goji concentrate, Stiebs goji puree, and Absolute Red goji by decreasing SOD activity by up to 76%, 68% and 73% compared to untreated cells. These results suggest significant suppression of reactive oxygen species production in HUVEC cells upon treatment with goji extracts.

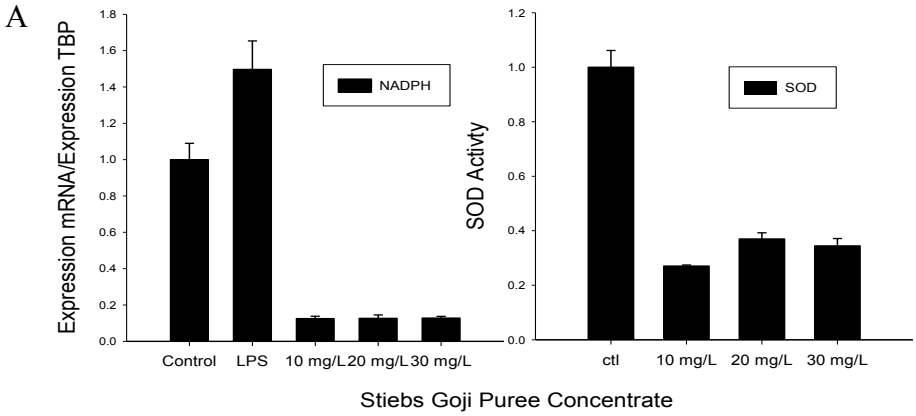


Figure 4.3 mRNA expression of pro-oxidant marker NADPH oxidase in HUVEC cells with 24hr LPS challenge (1μg/mL) and 30min pretreatment of goji extract. mRNA was analyzed by RT-PCR as ratio to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$). Activity of anti-oxidant enzyme Super Oxide Dismutase (SOD) in HUVEC cells with 24hr treatment of goji extract [A- Stiebs goji puree concentrate; B-Rich Nature goji concentrate; C – Stiebs goji puree; D – Absolute Red goji].

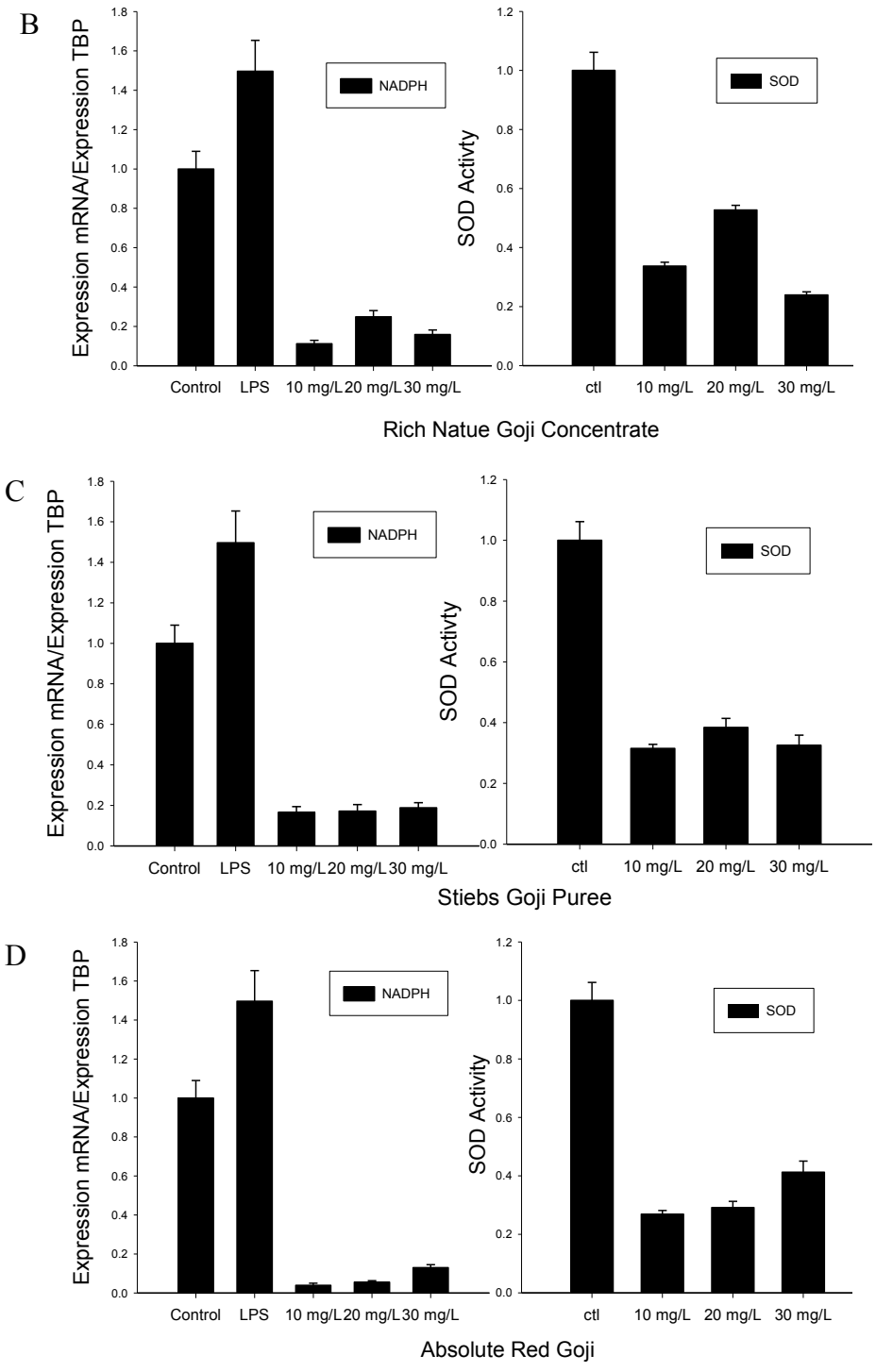


Figure 4.3 continued.

4.3.3.4. Anti-inflammatory effects of goji berry extracts

Rich Nature goji concentrate elicited the highest protective effect against inflammation compared to all tested goji extracts. The extract significantly lowered the LPS-induced increase of NF-κB, TNFα, IL-6 and IL-8 mRNA expression (Table 4.15). Rich Nature goji concentrate treatment doses of 10-30mg/L were used and results indicated higher treatment concentrations (>20mg/L) of the extract seemed to increase TNFα, IL-6 and IL-8 expression. This is likely due to the higher concentrations of phenolics inducing cell-stress. Rich Nature goji concentrate exhibited a decrease in mRNA expression of NF-κB, TNFα, IL-6 and IL-8 by up to 47%, 80%, 48% and 14%, respectively, compared to untreated cells. This suggests strong anti-inflammatory activity targeting the TNF inflammation pathway.

Stiebs goji puree concentrate significantly lowered the LPS-induced increase of NF-κB and TNFα expression in HUVEC. All treatment doses (10-30mg/L) elicited an inhibitory effect by up to 28% and 59%, respectively (Table 4.15). Treatment of HUVEC cells with Stiebs goji puree concentrate increased mRNA expression of IL-6 and no effect from the treatment was seen in mRNA expression of IL-8 (Figure 4.15B). These results would suggest anti-inflammatory activity upstream of NF-κB because suppression of NF-κB mediated proteins (IL-6 and IL-8) does not occur. There is a possibility that IL-6 and IL-8 are being suppressed on the protein level. Often, suppression of the pathway occurs post-transcriptional, meaning translation to the protein is inhibited but mRNA is still transcribed.

Stiebs goji puree and Absolute Red goji did not significantly affect the LPS-induced increase of NF-κB, TNFα, IL-8 and IL-6 expression (Table 4.15). Treatment of HUVEC with Stiebs goji puree (10-30mg/L) elicited up to a 19% decrease in mRNA expression of NF-κB, however an increase in mRNA expression was seen in the remaining biomarkers. Similarly, treatment of HUVEC with Absolute Red goji (10-30mg/L) elicited up to a 13% decrease in mRNA expression of TNFα, however no response was seen in the mRNA expression of NF-κB and IL-6. A significant increase was seen in mRNA expression of IL-8. This suggests the TNF inflammatory pathway

was not targeted and little to no anti-inflammatory activity was imparted with treatment of Stiebs goji puree and Absolute Red goji.

Table 4.15 Table indicates the mRNA expression of pro-inflammatory markers (A) NF-κB and TNFα and (B) Il-6 and IL-8 in HUVEC after a 24hr LPS-challenge (1μg/mL) in untreated HUVEC (control) and a 30min pretreatment with goji extract (treated cells). The treatment (10-30mg/L) corresponds to the extract listed in the first column. mRNA was analyzed by RT=PCR as ration to TaTa Binding Protein (TBP), data are mean ± SEM ($n \geq 3$) .

A

	NF-kB		**TNFa**	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Stiebs Goji Puree Conc.	2.00 ± 0.13	1.43 ± 0.09	69.32 ± 13.18	28.14 ± 4.80
Rich Nature Goji Conc.	1.71 ± 0.07	0.9 ± 0.04	63.62 ± 5.06	12.62 ± 0.82
Absolute Red Goji	1.82 ± 0.11	1.96 ± 0.07	33.11 ± 3.32	28.85 ± 0.27
Stiebs Goji Puree	2.24 ± 0.01	1.82 ± 0.06	33.11 ± 11.40	46.53 ± 1.74

B

	IL6		***IL8***	
Fruit Treatment:	***Untreated, control***	***Treated with extract***	***Untreated, control***	***Treated with extract***
Stiebs Goji Puree Conc.	4.7 ± 0.38	4.63 ± 0.8	34.2 ± 1.83	47.60 ± 1.85
Rich Nature Goji Conc.	3.15 ± 0.07	1.64 ± 0.07	34.69 ± 2.47	29.76 ± 0.13
Absolute Red Goji	4.7 ± 0.38	4.53 ± 0.17	34.69 ± 2.47	51.70 ± 4.76
Stiebs Goji Puree	4.7 ± 0.38	5.04 ± 0.14	34.69 ± 2.47	80.04 ± 15.02

The anti-inflammatory and antioxidant activities are likely due to the high content of phenolic acids and flavonoids, however aside from a select few, most are unidentified. The extracts also contained a large amount of poly-glycosylated compounds identified through HPLC-MS, which as discussed previously, may be the polysaccharide-protein complexes bound to phenolic compounds.

The secondary plant compound composition of goji would lead one to suspect anti-inflammatory activity. The results from this screening would suggest otherwise for

three of the four extracts. The two main compounds identified are di-caffeoylquinic acids and poly-glycosylated compounds. Di-caffeoylquinic acids have been studied using *in-vitro* and *in-vivo* assays confirming the bioavailability. However, there are isomers of this compound based on the location of the esterified caffeic acid – carbon number 1 or 3. While there is likely a mixture of these isomers in the goji extracts, it could potentially affect the anti-inflammatory activity through bioavailability. Another consideration is the effect of poly-glycosylated compounds on other phenolics in the extracts. As these compounds are not well characterized, their activity in terms of promotion or inhibition of activity of other phenolics has not been well researched. These results merit further research investigating the activity of the major compounds found in goji and their cooperative or suppressive effects of their bioactivity.

The bioactivity of Rich Nature goji concentrate elicited a stronger anti-inflammatory effect than the other goji products. The TPC of Rich Nature goji concentrate (1,824mg/L) was much lower than Stiebs goji puree concentrate (5,482 mg/L), Absolute Red goji (3,120mg/L), and Stiebs goji puree (3,228mg/L). Also, the predominant compounds found in this extract were unidentified poly-glycosylated compounds (332.59mg/kg). Similar chemistry was identified in the Absolute Red goji extract, however, this extract elicited little to no anti-inflammatory activity. Stiebs goji puree concentrate had a much higher TSP content than Rich Nature goji, however did not elicit as strong of a protective effect. A possible reasoning for this could be because the extract contains such a high concentration of the phenolic compounds it decreases the availability of the compounds *in-vitro.*

4.4. Conclusion

This study was a screening of exotic fruits conducted to increase available information regarding their secondary plant compound composition and the anti-inflammatory and antioxidant effect. Through an increase in knowledge of potential health benefits, a lucrative marking potential may be generated. CVD is a leading cause

of death worldwide, and with the current consumer interest in health and well being, these fruits can be marketed as possible preventative measures.

The results of this study were in agreement with that of previously reported literature investigating the phytochemistry of pomegranate and açai. Goji had few reports characterizing the phenolic composition. In fact, this was the first account of di-caffeoylquinic acids found in goji berry. There has been a plethora of research investigating the antioxidant activity and the effect towards CVD of pomegranate and açai. Goji, a fruit which is fairly new to the "superfruit" spotlight, has not been researched in relation to CVD. This study was the first to investigate the antioxidant and anti-inflammatory activity of the fruits in relation to preventative effects of CVD. The results of this study suggested significant anti-inflammatory and antioxidant activity of the screened exotic fruits. Antioxidant activity was seen in all fruits through a suppression of NADPH oxidase as well as a decrease in activity of SOD enzyme. Extracts of pomegranate grown in the China and Turkey, as well as both açai varieties elicited the largest decreases in mRNA expression of NADPH oxidase compared to untreated cells. Extracts of pomegranate grown in the China, Turkey and the US, as well as all goji extracts elicited the highest suppression of SOD enzyme activity compared to untreated cells. Reduction in mRNA expression of selected pro-inflammatory biomarkers (NF-κB, TNFα, IL-6, and IL-8) was seen in all fruits except Stiebs Goji Puree and Absolute Red Goji. The highest suppressive effect of the biomarkers was seen with treatment of pomegranate extract from India.

This investigation provided knowledge on the potential health benefits of açai, pomegranate, and goji. The results of this study are suggestive of anti-inflammatory and antioxidant activity, however further studies need to be completed relating the presence of the bioactive compounds to their bioavailability. These results also merit further investigation to determine the bioavailability of the secondary plant compounds found in these fruits. This knowledge provides a strong marketing potential for the selected exotic fruits, however the post-harvest handling and processing are still large issues regarding import to the United States, which needs to be studied in depth.

5. CONCLUSIONS

The health food industry has experienced considerable growth in recent years as the public interest in functional foods has grown. Consumers are looking for ways to improve their overall health and wellbeing through preventative measures and many are looking to the dietary supplement industry as well as the beverage industry to supply such functional foods. The fruits in this study are ideal candidates for these industries, as they are rich in secondary plant compounds giving them high potential for being beneficial to one's health.

The scope of this study was to determine possible anti-inflammatory and antioxidant effects of selected fruits. Inflammatory processes may lead to many outcomes, one of which being atherosclerotic lesions – a common manifestation of CVD (*3-5*). One of the leading causes of death in the United States, CVD is of great concern to Americans. By investigating anti-inflammatory effects targeting vascular lesions, the selected fruits could potentially be used as a preventative measure to CVD. The diagnostic measures defining Metabolic Syndrome have been correlated to the same risk factors leading to CVD. An increase in fruit intake would not only decrease the risk of developing vascular lesions through antioxidant and anti-inflammatory activities, but the dietary changes would likely reduce the risk of developing Metabolic Syndrome – further decreasing the risk of CVD.

Peach and plum fruits are widely consumed throughout the United States. Previous studies generated a need for investigation of potential health benefits (*13, 14, 35, 92*). Our results were in congruence with previous studies, indicating high antioxidant capacities – more so in the plum cultivar than the peach cultivars. *In-vitro* assays went on to confirm antioxidant activity quenching hydrogen peroxide induced reactive oxygen species in endothelial cells. This led to investigation of the pro-inflammatory mechanisms leading to vascular lesions. Results indicated no effect of the peach and plum extracts on LPS-induced receptor TLR-4, as well as cell-signaling mediators IRAK-1 and TRAF-6. This could be a result of the peach and plum extracts targeting a

different pro-inflammatory mechanism leading to NF-κB activation or the biomarkers may respond to LPS stimulation at a different time than when the biomarker expression was experimentally measured (3hr and 24hr). Pretreatment of LPS-challenged endothelial cells did significantly reduce the expression of NF-κB, IL-6, IL-8, V-CAM, and COX-2. Suppression of these pro-inflammatory biomarkers suggests a decrease in inflammatory activity in endothelial cells. Recent literature has shown a connection between the antioxidant activity and anti-inflammatory mechanisms involved in propagation of vascular lesions (*1, 2*). Because of the active *in-vitro* antioxidant and anti-inflammatory activity of peach and plum extracts, these fruits are ideal candidates meriting further investigation to be used as a dietary supplement as a preventative measure towards CVD.

Minimal research on a majority of the exotic fruit selections leaves information regarding biological activities leading to health benefits lacking. While fruits such as açai and pomegranate are well established, goji is still in the early stages of research. Chemical composition of pomegranate and açai fruits has been investigated in depth, however the characterization of goji berries focuses mainly on the protein-polysaccharides and not the secondary plant compounds. Phenolic characterization agreed with that of reported literature, aside from the identification of di-caffeoylquinic acid in goji berries. The results of this study suggest significant anti-inflammatory and antioxidant activity through suppression of pro-inflammatory and pro-oxidant biomarkers. Extracts of pomegranate grown in the China and Turkey, as well as both açai varieties elicited the largest decreases in mRNA expression of NADPH oxidase compared to untreated cells. Extracts of pomegranate grown in the China, Turkey and the USA, as well as all goji extracts elicited the highest suppression of SOD enzyme activity compared to untreated cells. Reduction in mRNA expression of selected pro-inflammatory biomarkers (NF-κB, TNFα, IL-6, and IL-8) was seen in all fruits except Stiebs Goji Puree and Absolute Red Goji. The highest suppressive effect of the pro-inflammatory biomarkers was seen with treatment of LPS-induced HUVEC cells with extract of pomegranate grown in India.

The mechanisms defining the relationship between anti-inflammatory and antioxidant pathways are still undetermined, creating a need for further clinical studies to demonstrate their bioavailability and activity *in-vivo* for use as a protective agent against CVD. While understanding the secondary plant compound composition and potential health benefits of the selected exotic fruits is a large step towards increasing the marketability as health-beneficial foods, there are many hurdles still ahead. Understanding genetic, seasonal, postharvest, processing, and import/storage conditions are imperative for the fruit to be used to its full potential. The fruits are generally marketed towards their health benefits and by defining these mechanisms a more targeted marketing approach can be taken.

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VITA

Name:	Emily Durham Townsley
Address:	1500 Research Parkway, Centeq 238A College Station, TX 77843
Email Address:	emtownsley@gmail.com
Education:	B.A., Chemistry, Texas A&M University, 2008 M.S., Food Science and Technology, Texas A&M University, 2010