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Dean, Graduate School of Nursing/Date

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THE EFFECTS OF BLACKCURRANT AND BERRY EXTRACTS ON OXIDATIVE
STRESS IN CULTURED CARDIOMYOCYTES AND MICROGLIAL CELLS: A
POTENTIAL CARDIOPROTECTIVE AND NEUROPROTECTIVE MECHANISM

by
MAJ(P) Tanisha L. Currie

Dissertation submitted to the Faculty of the
Daniel K. Inouye School of Nursing Graduate Program
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In partial fulfillment of the requirements for the degree of
Doctor of Philosophy, October 22, 2021

****for Dissertation Approval Form (paste here)**

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This is for my parents Vincent and Marcella Currie, brother Vincent Currie, family, friends, and mentors that have supported me throughout my entire professional and personal life journey. To my mentees and colleagues, we all share in this mission of optimized Soldier and Civilian health...this dissertation is especially for you. You have been a front and center motivator for me desiring to focus on nutrition and readiness for my area of study.

Thank you.

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The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: The Effects of Blackcurrant and Berry Extracts on Oxidative Stress in Cultured Cardiomyocytes and Microglial Cells: A Potential Cardioprotective and Neuroprotective Mechanism is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

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A black rectangular box redacting the signature of the author.

Tanisha Currie

October 18, 2021

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ABSTRACT

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality for both men and women in the U.S, and our military Warfighters are not immune to its increasingly harmful impacts. Our Warfighters are consistently placed in harm's way due to their mission of deterring the enemy's threats and protecting our homeland security. These high-stakes operational demands bring stressors related to military deployment, physiological changes related to cellular brain and heart health, which may also impact operational readiness. Oxidative stress is a key underlying factor in conditions of atherosclerosis and cognitive decline. Oxidative stress occurs at the cellular level when there is an imbalance of reactive oxygen species, reactive nitrogen species, and deficiency in antioxidant systems. Mounting evidence has suggested that flavonoids in the form of berry extracts may promote cell health through their antioxidant properties. Therefore, the effects of blackcurrant and various berry extracts were tested in two different cell lines - cardiomyocyte (HL-1) and microglia (BV-2) cells - to study their biological effects. The principal ingredients in blackcurrant and cranberry extract – delphinidin 3-rutinoside (D3R) and cyanidin 3-glucoside (C3G), respectively—were assessed as well. Our primary outcome measure was oxidative stress, but we also investigated effects on cell viability, inducible nitric oxide synthase (iNOS) expression, and nitric oxide (NO) production. The results showed that blackcurrant extract had similar antioxidant effects as N-acetyl-cysteine (NAC) in HL-1 cells in cellular protection and antioxidant effects, whereas cranberry extract was ineffective. In contrast, cranberry extract was comparable as an antioxidant to blackcurrant extract in BV-2 cells, but better-preserved cell viability, suggesting that cell viability may require more than just

antioxidant activity. Interestingly, blackcurrant and cranberry extracts showed divergent effects on Lipopolysaccharide (LPS)-induced iNOS induction in BV-2 cells. Blackcurrant extract appeared to sensitize the cells to iNOS induction with greater production of nitric oxide (NO), although cranberry extract suppressed iNOS induction. Both bioactive ingredients D3R and C3G reduced oxidative stress, which was similar to the berry extracts, indicating that these likely contribute to the antioxidant properties of the extracts. In summary, this investigation demonstrated that both blackcurrant and cranberry extract can reduce oxidative stress, but that they appear to work differently in different cell types. Overall, blackcurrant extract was more effective in the cardiomyocyte cell line consistent with cardioprotective effects, whereas cranberry extract was more effective in the brain microglia suggesting neuroprotective benefits. Lastly, berry flavonoids show promising effects as antioxidants and future research should focus on gaining a greater understanding of its nutrient application, dose-response, cell viability, and bioavailability in cellular and human models.

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LIST OF ABBREVIATIONS AND SYMBOLS

ASCVD (atherosclerotic cardiovascular disease)

BC (blackcurrant extract)

BV-2 (microglia cell model)

CR (cranberry extract)

CVD (cardiovascular disease)

C3G (cyanidin 3-glucoside)

D3R (delphinidin 3-rutinoside)

ROS (reactive oxygen species)

RNS (reactive nitrogen species)

FMD (flow-mediated dilation)

HL-1 (cardiomyocyte cell model)

Hs-CRP (highly sensitive C-reactive protein)

LDL (low-density lipoprotein)

MAOIs (monoamine oxidase inhibitors)

MDA (malondialdehyde)

NAC (N-acetyl-cysteine)

Ox-LDL (oxidized low-density lipoprotein)

PANAS-SF (Positive and Negative Affect Schedule)

PSS-10 (Perceived Stress Scale)

ROS (reactive oxygen species)

RNS (reactive nitrogen species)

RPM (revolutions per minute)

TNF Alpha (tumor necrosis factor-alpha)

CHAPTER 1: Introduction

INTRODUCTION

Over the last decade, cardiovascular disease (CVD) caused one out of three deaths in the U.S. and it continues to be the leading cause of death of Americans (13). In 2020, the American Heart Association reported \$351.2 billion were associated with direct costs of CVD; and \$137.4 billion due to lost work productivity (13). Both civilians and the U.S. Active Duty military force are experiencing increased CVD (148). Even young military personnel with recent combat experiences may be at an increased risk for the development of CVD compared to their non-military peers due to sleep disturbances, daily stress, post-traumatic stress disorder (PTSD), and heightened inflammatory responses (88; 89). In particular, military warfighters are consistently faced with challenges of deployment, marital separation, role strain, individual and family readiness, and are under great stress due to their intense operational demands (62). Chronic and intense operational demands harm psychological health, accelerate atherosclerosis development (inflammatory disease of the vessel wall within the heart), and are associated with weight gain (62; 89; 119; 135). These factors may decrease military readiness and performance (62). For instance, a recent Department of Defense (DoD) Health of the Force report showed that 16% of active component soldiers had one or more behavioral health diagnoses to include depression, anxiety, and post-traumatic stress disorders that influence psychological health (6). This report also shed light on the fact that psychological health within the active-duty population was the second leading cause of limited duty days impacting medical readiness and overall work productivity (6).

Therefore, it is even more crucial to understand the larger problem of CVD and its impact on brain health through its linked relationship of *oxidative stress* at its most foundational level. Currently, we lack strategies to address CVD and brain health at the cellular level within the military.

Underlying Mechanisms of CVD and Brain Health

CVD is a prevalent issue within our society, and the military is not exempt from its impact on health. An underlying mechanism for CVD is atherosclerosis.

Atherosclerosis is an inflammatory process that begins with endothelial dysfunction in the arterial wall (119). Endothelial dysfunction is the earliest and hallmark sign of lesion formation (119). Atherosclerosis in its nascent phase disrupts the homeostasis of the endothelium by depositing low-density lipoproteins (LDL) within the walls of the intima layers. This process signals oxidative stress responses, and recruitment of monocytes and macrophages to the area, causing further inflammatory processes (119; 145). As the pathophysiology advances in the atherosclerosis phase, foam cells develop and trigger the release of cytokines and interleukins, smooth muscle cells migrate into the arterial space, a fibrous cap forms over the lesion, and rupture of the lesion and thrombus formation can potentially occlude a coronary artery, obstructing blood flow to the myocardium (145).

The pathophysiological process of oxidative stress and inflammation linked to CVD is also concerning for brain health. Oxidative stress is generated when there is an overproduction of reactive oxygen species (ROS) and there is an imbalance between antioxidants and ROS (76). Similar to the adverse effects on the CV system, underlying mechanisms of oxidative stress and inflammation in the central nervous system (CNS) may contribute to decreases in monoamine neurotransmitters such as serotonin,

dopamine, and noradrenaline that support brain and psychological health (52).

Decreasing levels of monoamine neurotransmitters can lead to impaired psychological health such as depression (103). To mitigate these harmful psychological impacts, common pharmacological therapies such as monoamine oxidase inhibitors (MAOI) and selective serotonin reuptake inhibitors (SSRI) are typically prescribed by healthcare providers as pharmacological treatments to support imbalances in neurotransmitters and psychological health (157). Although these pharmacological treatments are effective at times, there are concerns of dietary interactions with its administration that can potentiate cardiovascular effects (hypertension) known as the “cheese effect” when there is an interaction between MAOI and tyramine; and concerns of serotonin toxicity (41). Therefore, more adjunct innovative approaches are needed to address this gap. As an alternative approach to pharmacological interventions for addressing CVD and brain health, scientists have begun to shift their attention to a potentially promising direction of research in the sciences of flavonoids.

FLAVONOIDS

Flavonoids are a diverse group of secondary metabolites within plants (69). Flavonoids are commonly found in food sources such as fruits, vegetables, teas, red wines, and chocolates (69). Flavonoids are composed of anthocyanins, which provide fruits, vegetables, and flowers pigments of red, blue, and purple hues (70). Additionally, anthocyanins such as cyanidin and delphinidin are naturally occurring bioactive ingredients within flavonoid-rich foods like berries, such as blackcurrants. Such foods have been associated with antioxidant and anti-inflammatory properties for cardio- and neuroprotective health benefits (69; 106). Specifically, scientists have reported flavonoid

actions on countering negative cellular effects of oxidative stress and inflammation, modulating cellular signaling pathways, and improving vascular health (120). Moreover, additional research has focused on its role in brain health, nutrition, and relationship to microglial cell activation. The data suggest increased production of ROS and induced nitric oxide synthase, cytokines, and inflammatory processes (131; 134). Since an association between inflammation and oxidative stress has been linked to psychological health such as negative mood, it is even more imperative to seek strategies to close these relevant gaps that may impact health readiness (71). Essentially, there is a paucity of research investigating the role of berry flavonoids, and in particular blackcurrant extract in cellular models. Blackcurrant has a potent antioxidant profile due to its active primary ingredient, delphinidin 3-rutinoside (4; 118; 138). Further research is warranted in these cell lines to advance the science on flavonoids and oxidative stress mechanisms. This may have important implications for improving the health of individuals under high stress, including warfighters, by using alternative approaches such as dietary supplements in conjunction with a whole food diet in garrison; or use as a dietary supplement where fresh fruits and vegetables are limited in austere environments to reduce oxidative stress.

Although this area of research is not fully elucidated, there is a baseline understanding of some of the mechanisms of action for flavonoids.

CELL MODELS

Cell models may provide an innovative way in which to investigate mechanisms of oxidative stress on CV and brain health that is complimentary to clinical studies. Through investigation of cell models that propagate underlying mechanisms of oxidative stress on CVD and brain health, potential solutions may be discovered to promote CV

and brain health. In addition, investigating the role of oxidative stress in cell models of cardiomyocytes (H1-1) and microglial (BV-2) cells, in particular, may provide a way to detect subtle changes in underlying mechanisms of atherosclerosis. Cell models have been found to play an instrumental role in indicating the status of cellular mechanisms of injury, disease processes, and drug discovery which may contribute to finding nutritional interventions for warfighter health. For instance, a team of scientists investigated the effects and dose-response of a dietary antioxidant, curcumin, on mouse skeletal C2C12 myoblasts and found that at different concentrations curcumin produced either mild or high levels of ROS and oxidative stress. At mild levels of oxidative stress, it was demonstrated that a low dose of curcumin (5 μ M) improved mitochondrial health (mitochondria mass), membrane potential, and protected cells from oxidative damage (158). Such work, in addition to defining an effect, shows the importance of dosing to be within a therapeutic window. However, when higher doses of curcumin (20 μ M) were administered to C212 myoblast cells, high levels of oxidative stress and promotion of cell death were demonstrated (158). This study also showed that curcumin at dose-dependent concentrations on C212 myoblast cells regulated ROS, and curcumin provided a biphasic response of cellular protection or injury. As for berry flavonoids, which are commonly contained in fruits such as berries, studies have shown that they contain potent antioxidants and health benefits to improve CV and brain health (138). For example, in one study investigators used grape seed extract as a pretreatment within cardiomyocyte cells. Grape extracts demonstrated significant effects by decreasing oxidative stress in a dose concentration manner (106; 125). This study also indicates that grape seed extract has potential cardioprotective benefits. Similarly, within another study, a common berry

such as elderberry demonstrated the ability to regulate oxidative stress through inhibitory effects within microglial cells which have similar functions to macrophages, but within the brain (131). This research study showed the response of elderberry extracts through induced inflammatory cellular responses of inflammation and oxidative stress and found that elderberry at 1mcg/ml levels reduced both oxidative stress and inflammation within the microglial cells (131).

These studies suggest that various flavonoids found in plants, such as berry flavonoids may exert cardioprotective and as well as neuroprotective properties within various cell culture models. These are important considerations in examining the role of dietary antioxidants on health and understanding the potential benefit for ameliorating oxidative stress.

Therefore, the physiological role of oxidative stress and application to various cell models were explored to assess the mechanisms of actions for berry flavonoids and their impact on CV and brain health. The diagram below provides a conceptual model on the effects of stressors generating ROS and the possible role of berries in protecting warfighter health.

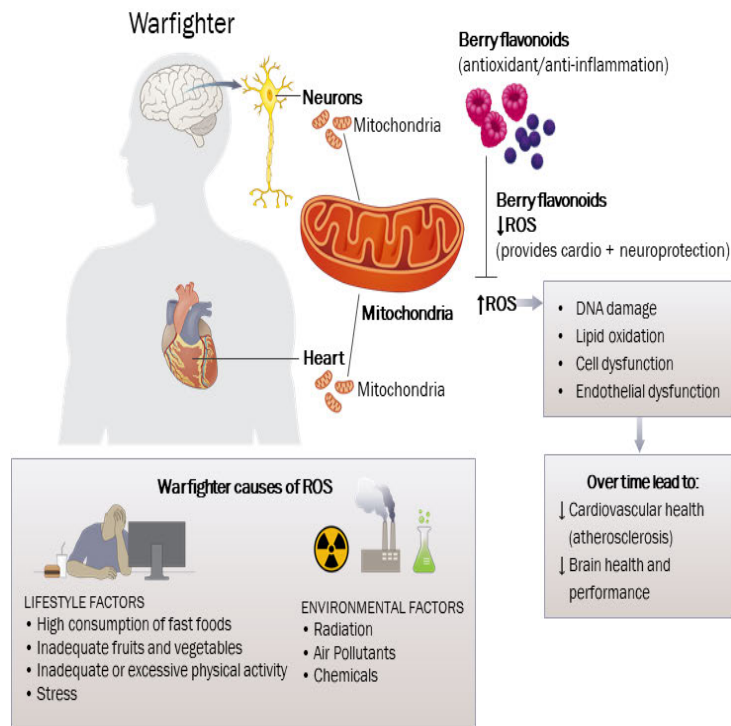


Figure 1. Effects of ROS on Warfighter Health

The figure above depicts how berry flavonoids can affect warfighter CV and brain health by decreasing ROS and oxidative stress in neural and cardiac mitochondria. Warfighter brain health and CV performance are essential for meeting the demands of operational readiness and military performance. Excess reactive oxygen species can damage these mitochondrial energy sources which can lead to compromised brain and CV health. Over time, this may also affect brain function and CV performance. Berry flavonoids with antioxidant and anti-inflammatory properties may offer a portable solution for warfighters to prevent the oxidative stress induced by environmental and work-related stressors.

THE ROLE OF OXIDATIVE STRESS

Oxidative stress refers to the imbalance of reactive oxygen and reactive nitrogen species and a deficiency of antioxidants in the body (65). ROS was first discovered in phagocytes as part of the defense response. ROS are now known to have multiple roles in cell signaling. Nitric oxide (NO), a potent vasodilator, is affected by oxidative stress. Under physiological conditions, NO relaxes blood vessels and decreases blood pressure (155; 156). NO is produced by the enzyme endothelial nitric oxide synthase (eNOS) which plays a critical role in inhibiting platelet aggregation, cellular wall adhesion of leukocytes, and inhibition of low-density lipoprotein (LDL) (156). When oxidative stress becomes a prolonged process, it triggers unstable oxygen molecules, which causes a reduction in NO and leads to endothelial dysfunction; this can result in CVD (65; 156). In addition, a relationship between endothelial dysfunction and depression has been postulated (49). A reduction in NO production and increase in cellular adhesion of inflammatory cytokines and leukocytes may potentially result in inhibition of neurotransmitters such as serotonin, and thereby lead to depression and microglial activation (49; 134). Therefore, applications utilizing a cardiomyocyte-derived cell model (HL-1 cells) and microglial cell model (BV-2 cells) to investigate the effects of berry flavonoids on oxidative stress may provide important information about the potential protective role of these compounds in CV and brain health.

Cell Model Applications for Mechanisms of Oxidative Stress in CVD and Brain Health

HL-1 Cardiomyocyte Cells

HL-1 Cardiomyocyte cells are a common cardiac muscle cell line derived from the atrial cardiac muscle of a transgenic mouse (25). HL-1 cells are produced by an expression of the Simian virus (SV40) large T antigen from a cardiomyocyte tumor lineage (25). HL-1 cells have been proven to be beneficial in studying cellular signal processes such as oxidative stress, apoptosis, endothelin-1, and cardiac effects of pharmacological agents. In addition, HL-1 cells are also useful in studying other areas of cardiac biology such as hypoxia, hyperglycemia, and electrophysiology (112; 152). Overall, pharmacological interventional studies with HL-1 cells for various CVD states have been more aggressively researched in this cell line as opposed to investigating alternative nutritional approaches to CV health. Currently, there is a clear lack of research investigating HL-1 cells and their application in oxidative stress processes by using different nutritional or dietary supplement approaches to improve CV health. Applications of an HL-1 cell model to investigate a mechanism involved in atherosclerosis, in particular oxidative stress, with a dietary supplement approach may provide a proof of concept for conducting further human studies. In addition, this investigation may also provide a dietary supplement strategy for military service members facing austere environments who are exposed to stressful conditions that induce high levels of oxidative stress.

BV-2 Microglial Cell Line

Microglia are resident brain immune cells that play a pivotal role in helping to maintain a healthy central nervous system (CNS) (7; 117). Microglia have a specialized role in the CNS that mimics the function of the body's peripheral macrophages. The microglia is considered the brain's first defense or immune cells of the CNS. Microglia can sense infection or injury within the CNS, phagocytose and remove dead cells (117). In addition, they release inflammatory cytokines/chemokines, ROS, and NO as part of their immune response to infection and injury. Activated microglial cells may also indicate an infection/inflammation or need for neuronal protection; this is the opposite of resting healthy microglia of the brain that are not activated (117).

The original "microglia-like" cell or (BV-2) cell line was developed to study the microbiology of the brain and detect and evaluate the effects of inflammation, oxidative stress, and various treatments. (144). BV-2 cell models were generated by infecting a mouse microglial cell culture with an oncogene J2 virus which can exhibit inflammatory properties (14). BV-2 cells are an immortalized cell line with phagocytic cells originating from the bone marrow of a mouse (14; 142). BV-2 cells are of interest to the current study due to their simulation of cellular brain responses and the feasibility of evaluating a dietary supplement on cellular mechanisms of oxidative stress leading to neuroinflammation. Some cell studies have evaluated flavonoids and their impact on cellular inflammation and oxidative stress; in particular, a study tested tart cherry on a microglial cell model and showed that tart cherry significantly reduced inflammation, oxidative stress, and NO expression (130). Another study investigated human pretreated serum *in vitro* with strawberry and blueberry extracts and their effects on rat HAPI (highly aggressively proliferating immortalized) microglial cells (120). They found that

45 to 90-day consumption of the strawberry or blueberry extract significantly reduced LPS induced inflammation, NO, and TNF-alpha, COX-2 at different time points (120). These studies combined provide evidence that berry flavonoids can modulate cellular signaling processes, reduce inflammatory and oxidative stress markers in the presence of an insult. Additionally, these studies provide evidence that berry extracts may provide neuroprotection through their free-radical scavenging properties that are also associated with optimized brain health.

The study sought to discover the differences between berry flavonoid profiles and their neuroprotective properties. This is useful for military relevance and applications to optimizing brain health by finding solutions that may reduce or inhibit microglial activation from oxidative stress. In the following section, flavonoids and their link to cell models were explored.

Flavonoids and Cell Models

Epidemiological studies demonstrate an association between fruit and vegetable intake and lower risk of CVD, due to their phytochemical compounds known as flavonoids (87). Flavonoids are found in several food sources such as berries, teas, and blackcurrants. They exert anti-inflammatory and anti-platelet effects in endothelial cells as well as reduce lipid peroxidation (39; 87). Of particular interest are flavonoid-rich blackcurrant byproducts or metabolites because of their ability to cross the blood-brain barrier and act as MAO-inhibitors (150). This provides potential neuroprotective, cognitive, and psychological health benefits (150). Although the impact of berry-derived fruit extracts on CV and brain health may not be fully elucidated, there is growing evidence about their mechanisms of action (23; 52; 66). Additionally, the impact of

flavonoids on gut and immune health has gained greater attention. Scientists postulate that after consuming berry flavonoids, their antioxidant and anti-inflammatory properties may modulate the gut microbiota (109). Berry flavonoids' effect on the gut microflora may occur by increasing the good bacteria of lactobacillus within the intestine (109). In a recent study, the effects of berry flavonoids were studied in a model of ischemia-reperfusion injury in mice intestines. This study reported that the combination of berry flavonoids such as chokeberry *in conjunction* with Lactobacillus Plantarum HEAL 19 (LplH19), a probiotic strain, demonstrated a significantly higher ($p=0.029$) viable count of mucosal tissue in comparison to the chokeberry (only)-fed mice group (59). This supports evidence that flavonoids may have a synergistic effect using the body's "good bacteria" to support gut and immune health. As for potential effects on brain health, another study used elderberry extracts, which commonly contain cyanidin 3-glucoside, on a microglial cell line (59). The results showed that elderberry at 400 ug/ml significantly reduced LPS and interferon ($IFN\gamma$) induced oxidative stress [$p < 0.01$ and $p < 0.001$ respectively] (131). Furthermore, the elderberry extract dose concentration of less than 400 ug/ml in this model was found to be less effective as a dose concentration on induced oxidative stress (131). Additionally, this study reported that cyanidin 3-glucoside as an individual active ingredient of elderberry extract, was able to significantly decrease LPS and $IFN\gamma$ -induced ROS production at concentration doses of 6.25, 12.5, and 25 ug/ml in the BV-2 cells (131). Therefore, elderberry extract and its active ingredient compound, cyanidin 3-glucoside have potent antioxidant or free-radical scavenging activity that suppressed LPS and $IFN\gamma$ -induced ROS production at optimal dose concentrations. Overall, these cell studies demonstrate that berry flavonoids have antioxidant potential in

various cell models. This suggests that berry flavonoids may also have general ROS systemic effects in the human model. Further investigations are warranted to examine the effectiveness of berry flavonoids in cell model systems of oxidative stress.

Background and Significance

CVD is associated with atherosclerosis and the key initiating event is endothelial dysfunction. As the disease progresses and atherosclerotic plaque develops, the plaque ruptures, and the resulting thrombus increases or occludes coronary artery blood flow to the myocardium. The causal theory of atherosclerosis is attributed to oxidative stress and inflammation (119). This disruption in homeostasis causes endothelial dysfunction in the arterial vasculature system. The oxidative stress and inflammatory processes may also affect the central nervous system (CNS) which leads to changes in brain health. Interventions that prevent or reverse these pathophysiological mechanisms associated with atherosclerosis and CVD such as *oxidative stress* may improve vascular health and decrease the risk of CVD. For example, statins are cholesterol-lowering drugs and are the most commonly prescribed medication for lowering cholesterol and subsequent risk of coronary artery disease. Although statin drugs have been proven to be effective, there are potential concerns in the military force for its adverse effects after prolonged consumption (113). These include debilitating muscle conditions such as myalgias and rhabdomyolysis as well as other adverse effects such as liver damage and Type 2 diabetes that may limit the use of statin drug therapy in some individuals (113). Therefore, novel and risk-mitigating solutions are needed to address this CVD problem both from a preventative and an interventional vantage point within the military community. Given

the results of experimental studies, nutrients with flavonoids may have the potential to improve CV health.

Flavonoids are classified as polyphenols with a 15-carbon structure (C6-C3-C6) (106) and are found in fruits, vegetables, teas, berries, wine, and chocolate (12). They provide purple, red, and blue pigmentation in flowers and fruit sources (106). Flavonoids have demonstrated anti-oxidative, anti-inflammatory, cardioprotective, and neuroprotective properties (12; 106). However, it is essential to understand the cellular mechanisms for berry flavonoids, and cardiac and neural cell lines as indicators of CV and brain health. The crucial gap in this area of science is the limited research in cell lines with flavonoid-rich nutrients such as blackcurrant extract and its effects on oxidative stress. These in-vitro models serve as simple screening systems of biochemical effects that will lead to clinical intervention trials.

In cardiomyocytes and microglial cell studies, flavonoids decrease oxidative stress and inflammatory responses in vitro (92; 130). Human and animal studies with flavonoids have demonstrated effects of lower lipid levels, improved blood pressure, increased flow-mediated dilation, and enhanced mood and/or cognitive performance (36; 66; 102). There are a few advantages to pursuing this in vitro research investigating berry flavonoids and their bioactive compounds on outcomes of oxidative stress. First, cell-based research can provide valuable data on berry extracts' responses to oxidative stress within various cell models. Second, research data can be procured in a relatively shorter time frame with dietary supplement interventions to test specific supplement efficacy and effectiveness in a cell model versus human model systems that may take longer. Third, cell models provide information on berry extract dose ranges that can be used to inform

future human studies. Fourth, various outcome measures can be performed to detect cellular signaling pathways that may be involved in specific cell responses. This current cell study provides information on the effects of various berry extracts and bioactive compounds on primary outcome measures of oxidative stress that can potentially lead to detrimental effects to warfighter health, if not balanced with proper dietary antioxidant proportions. Furthermore, this investigation adds to the body of science about the effectiveness of berry extracts at various dose ranges. However, more research is critically needed at the cellular level to explore the mechanisms associated with oxidative stress. It is also important to investigate the individual chemical constituents of the berry extracts by testing their effects on oxidative stress in vitro. Reducing oxidative stress in the military population may decrease future chronic conditions of CVD as well as maintain and protect brain health. The relationship between oxidative stress and berry flavonoids will be addressed in the following section. Moreover, the ingredients found in flavonoids that may promote health will also be discussed.

OXIDATIVE STRESS AND BERRY INTERVENTIONS

Oxidative stress indicates an imbalance of ROS and reactive nitrogen species (RNS) and decreased antioxidant intake (67). ROS precedes oxidative stress when unpaired electrons contribute to free radicals. ROS and RNS are chain reactive, work together to damage cells within the body, and cause a breakdown in NO production (11; 139; 156). Once NO function is impaired, leukocyte and platelet aggregation and adhesion may occur (156). Adverse vascular effects occur including less vasodilation, decreased blood pressure, and lipid peroxidation at the vascular wall (156). In addition,

ROS-induced damage can also affect cell membranes and the myelin sheath of neurons (139). This may lead to the development of chronic conditions such as CVD, neuropathies including multiple sclerosis, and cancer (136). As ROS continues to overburden and alter homeostasis in the body, oxidative stress may be responsible for chronic conditions such as CVD (65).

Mounting evidence has focused on nutritional interventions such as flavonoids to decrease oxidative stress by reducing ROS and RNS production. For example, in a randomized-controlled, parallel study, participants were given 480 ml of tart cherry juice, a rich source of flavonoids, or a control drink daily for 12 weeks. From a sample of 37 women and men, the experimental group showed significance in DNA repair, decreased C-reactive protein (CRP), and low-density lipoprotein (LDL) levels (21). There was no significant effect on oxidized LDL (Ox-LDL) levels. The decrease in CRP levels and DNA repair are suggestive of a reinforced antioxidant response at inflammatory pathways and cellular protection as a result of tart cherry supplementation (21). Similarly, in an earlier study, blackcurrant (high or low concentrations) was provided to 44 healthy adult research participants in a six-week randomized, double-blind, placebo-controlled, parallel study (67). The study was carried out to assess whether blackcurrant, rich in vitamin C and flavonoids, was able to increase flow-mediated dilation (FMD) and produce significant changes in oxidative stress blood markers (67). Results demonstrated that the high concentration blackcurrant group increased FMD, and decreased oxidative stress biomarker F₂-isoprostanes in both the low-and high-concentration blackcurrant groups (67). These findings underscore the potential critical application of fruits high in flavonoids in decreasing oxidative stress and improving endothelial function and blood

flow. Additionally, another key aspect to understanding the interplay between oxidative stress and the effects of berry flavonoid interventions is consideration of their primary active ingredients that may contribute to the observed properties of berry flavonoids.

BERRY FLAVONOIDS: COMMON ACTIVE COMPOUNDS

Berries provide a rich source of anthocyanins and flavonoids that give the characteristic vibrant blue, purple, and red hues in fruits. Berry flavonoids also have antioxidant and anti-inflammatory properties. Depending upon the berry fruit origin, seasonal conditions, preparation, and primary ingredients, berries can possess different antioxidant potential. In particular, various berry flavonoids such as those found in blackcurrant, elderberry, aronia berry, cranberry, cherry, and berry blends can have different primary active compounds with differing free-radical scavenging activity. Since not all berries have the same antioxidant potency against oxidative stress, it may prove beneficial to explore the effects of primary active compounds from various berries. This will help determine the antioxidant potential for specific flavonoid-rich berries. For example, blackcurrant commonly contains four major active compounds including cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside, and delphinidin-3-rutinoside (17). These ingredients in combination have been linked in research to provide both CV and neuroprotective properties (17). Interestingly, there are limited studies in the area that have researched specific individual berry active compounds utilizing the cardiomyocyte or microglial cell model lines. By understanding the antioxidant potential of the active components in berry flavonoids in specific cell lines, the distinction in antioxidant potency of individual compounds may be determined. For example, blackcurrant extract is composed primarily of delphinidin 3-rutinoside (D3R). In one

study, investigators examined the effect of D3R on oxidative stress in the MC3T3-E1 or osteoblastic cell model for the prevention of osteoporosis (18). The results showed that D3R was able to provide a protective effect by inducing cell viability (18). In addition, D3R as a pre-treatment significantly reduced tert-butyl hydroperoxide (t-BHP) induced-oxidative stress in the osteoblastic cell model (18). In another study, D3R was studied in bovine ciliary smooth muscle. Investigators focused on decreasing excessive endothelial or ET-1 (potent vasoconstrictor) contraction of the eye (85). D3R inhibited ET-1 induced contraction which suggests that D3R may have the potential to increase NO production leading to bovine ciliary smooth muscle relaxation (85). These two studies with specific cell lines provide evidence that a dominant flavonoid strain in blackcurrant, such as D3R, may play a key role in reducing oxidative stress and increasing NO production.

Therefore, it is of interest to study D3R in other cell lines to understand its potential health benefits since it has potent antioxidant properties in these models.

Another more common active compound that may be found in other berry flavonoids is cyanidin 3-glucoside (C3G). It is present in blackcurrant as well as elderberry, aronia berry, cranberry, cherry, and berry blends. In a recent review article, C3G was most frequently identified as a useful biomarker after consumption and was detected in both human urine and plasma out of a total of 203 berry flavonoid compounds assessed (122). C3G was detected in 69% of human plasma samples and 58% in urine samples after consumption as a berry flavonoid (122); C3G has also been detected in the vascular endothelium and brain (122). This points to evidence that C3G is absorbed and taken up into the body's tissues and can cross the highly selective blood-brain barrier. Hence, C3G may be more bioavailable, and this explains why it has been studied more in

humans, animals, and cells relative to other berry flavonoid ingredients. Similar to D3R's effect on ROS, C3G has antioxidant properties through its reduction of oxidative stress (140). Additionally, C3G has been reported to have positive benefits in the gut microbiota once absorbed in the gastrointestinal system. Scientists postulate that C3G may have inhibitory effects on epithelial inflammation and it supports intestinal health through modulation of the gut microbiota (140). C3G may be an ideal active compound to investigate due to its antioxidant potential. It may also serve as an important comparison to D3R which is an active independent ingredient in other cell lines.

THE PILLARS OF NUTRITIONAL READINESS FRAMEWORK

A conceptual model on flavonoids and military lifestyle considerations is presented below in Figure 2 as it relates to antioxidants, potential health benefits, and operational readiness. The figure depicts a broad scope perspective across the nutrition landscape within the military environment. First, the military population is growing increasingly challenged to stay physically fit and operationally ready in an evolving (blue box) proinflammatory environment; given its easy access to fast-food restaurants and convenient marts that have increased across all military installations. Additionally, the military force is facing similar “westernized” dietary concerns on the effects of ultra-processed and processed foods on heart and brain health. However, there is a need for increased nutrition education that is targeted for the Warfighter beginning at the accession point of their career and to their exit point as a veteran. Second, the grey box reflects the need to develop strategies from the unit to the leadership level, as well as with healthcare providers to encourage a culture of health within the military nutrition landscape. Third, the green box connects the operational mission of health readiness to

practical strategies of equipping the Warfighter with nutrition practices (increasing flavonoid consumption) that may enhance heart and brain health. Lastly, the purple box depicts how this current research seeks to increase Warfighter health through investigating the benefits of berry extracts and their constituent ingredients at the cellular level; and eventually providing research relevant data that may impact Warfighter “portable” dietary considerations in the future.

The model provides a conceptual framework for the barriers to optimized nutrition within a military context. This framework provides a big picture perspective for future human study investigations with a military nutritional lifestyle approach using a berry flavonoid to improve CV and brain health.

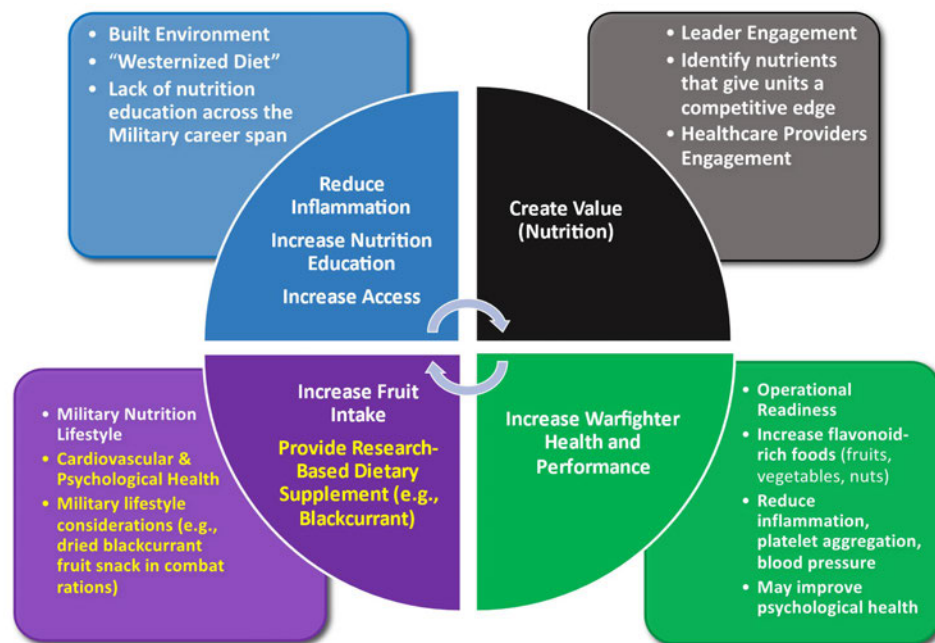


Figure 2: Pillars of Nutritional Readiness

This provides a framework for nutritional readiness across the military landscape.

MILITARY LIFESTYLE CONSIDERATIONS AND DIETARY SUPPLEMENT USE

The military community must continually be alert due to its mission of readiness, which causes potential stressors that negatively impact health. Flavonoid-rich berries such as blackcurrant, as a dietary supplement, may provide potential health-promoting benefits for this at-risk community. The military culture is faced with unique operational demands and lifestyle stressors (62; 110); in which military personnel are tasked with ensuring they are physiologically and psychologically fit. In a recent longitudinal study, Jayne et al. (year) used 48 months of data from the Stanford Military Data Repository and reported there was a relationship in active duty military soldiers' health status with stressful life changes and nutrition-related outcomes (62). For example, factors of marriage, adding a child, military separation, deployment, etc. negatively impacted the soldier's lipid profile, led to increased weight gain, and was associated with nonmedical readiness (62). The military lifestyle differences and additional occupational demands provide a contrast to the civilian population as it relates to nutritional considerations for the military warfighter. In another study, Lieberman et al. found that due to the military's intense requirements of readiness, more than 50% of military personnel consume dietary supplements to better their health and performance (74). Other primary reasons for consuming dietary supplements were reported to promote general health, enhance performance, increase endurance, energy, and weight loss (74). Moreover, the study demonstrated that although younger soldiers consume dietary supplements, the active-duty older population (age ≥ 40) was more likely to consume them at a greater rate. This older active-duty population includes individuals who attained advanced education and were more likely to be a part of the Special Forces community (75). Although there are

concerns with the use of dietary supplements in this community, largely surrounding these concerns are related to Soldiers using nonresearched based supplements, lack of dietary supplement education for the military, and the unpredictable contents of proprietary blends (32). Since dietary supplements are of high use within this particular population, a research-based dietary intervention to supplement whole food diets may fit within the military lifestyle to promote CV and psychological health.

Military Stressors

Personal and environmental stress factors related to military lifestyle may increase oxidative stress and inflammation, which are associated with a rise in CVD and impaired psychological health, making our warfighters less medically ready for deployment(35; 62; 128). Indeed, warfighter medical readiness continues to be negatively impacted by the rise in CVD (24; 105). Examples of health and occupational determinants in the military that may lead to higher CVD prevalence include tobacco use, lack of access to fresh fruits and vegetables in deployed environments, and consumption of energy-dense (rather than nutrient-dense) diets (1; 35). Soldiers consuming a poor diet and experiencing negative environmental factors may be more prone to a pro-inflammatory state. Therefore, based on current experimental and clinical studies, blackcurrant extract as a dietary supplement may offer an innovative, safe, and adjunctive therapy to standard care (e.g., statin drugs) to reduce inflammatory and oxidative stress processes that may be contributing to CVD and impaired psychological health.

Moreover, mental health is a complex topic, especially within the military culture. Addressing mental health concerns requires soldier vulnerability to confront the issue of impaired psychological health; this is a difficult ask for military personnel to be

vulnerable considering their operational fields, machismo culture, and varied leadership culture and acceptance. Therefore, it is not surprising that Wooten et al. (2018), discovered a high use of behavioral health services purchased outside military medical facilities (153). This report showed that the frequent and highest use of purchased care visits were due to mental health issues of PTSD, anxiety, tobacco use, opioid misuse, and mood disorders (153).

Psychological health and nutrition are emerging areas of science focusing on ways to improve mental health in areas such as depression, anxiety, post-traumatic stress disorder (PTSD), and other neuropsychiatric conditions (1). With the rise in depression and suicide statistics within the military (54; 127), seeking nutritional interventions to mitigate these rising rates may serve as a practical strategy for increasing mental resiliency. A poor diet can affect psychological health (44; 121), and blackcurrant extract may be a good way of providing antioxidants to soldiers to supplement a whole-food diet, when possible. Diverse solutions are needed to target mental health issues in the military community by using research-based strategies that will also address ways to reduce mental health stigmatization (51).

TRICARE and Dietary Supplement Considerations

Although dietary supplement use could enhance military health, there are limitations to the U. S. Military Health System, which could make integration of this nutritional strategy difficult. The U.S. Military Health System is responsible for delivering care to an estimated 9.5 million military personnel, retirees, their beneficiaries, and those eligible for DOD health care under the managed care system of TRICARE (141). TRICARE is essential to warfighters, retirees, and their families in need of medical

care. Understanding TRICARE's role as it relates to receiving direct care, accessing health services, securing high-quality care, and governing services provided is essential to informing research strategies and development. Currently, dietary supplements, such as blackcurrant extract, are not covered under TRICARE's plan. According to TRICARE's policy, individuals requiring nutritional therapy or "medical necessary food" intended to treat specific diseases or conditions would be covered under its current policy (147). Although this may seem to deter the research-based dietary supplement approach, it is important to understand that TRICARE has evolved to meet the needs of its consumers. Therefore, research-based strategies such as blackcurrant extract enriched with flavonoids, to supplement a soldier's diet may prove to be a worthwhile investigation that could be implemented in the future. A blackcurrant extract supplement may offer biochemical properties of anti-inflammatory and radical scavenging activities that may quell oxidative stress processes (28). Another military lifestyle consideration is that a blackcurrant supplement comes in the form of a portable capsule, and there is high support of dietary supplements within the military culture amongst its soldiers (75).

Military Culture, Mental Health, and Berry Considerations

Since the military culture embraces mental and physical toughness characteristics; soldiers often display a level of stoicism and mission-first attitude (141) which can be perceived to leave little room for impaired psychological health. As psychological health and innovative solutions are considered for this community, it is important to consider how these strategies may fit within the military lifestyle. Increasing research has begun to emerge in the area of mood regulation and mental health. Currently, research is examining the association between food and mood and its relationship to brain health

(50). Whole fruits and vegetables are demonstrating positive health effects on brain health and well-being (27). In particular, flavonoid-rich berries may have an association with improved mental health (10; 66). However, as we consider military lifestyle, it may be beneficial to supplement a whole food diet with flavonoid-rich berries to supplement their diet. In addition, other benefits of a dietary supplement approach is reduced mental health stigmatization of the soldier, as traditional approaches and pharmacological interventions may have brought unwanted stigmatizations (51). A dietary supplement approach is within the military cultural norm. Although soldiers may require additional mental health services such as pharmacological and traditional approaches, a dietary supplement may not add to the mental health stigmatization (51). Therefore, an opportunity may exist in which a soldier's CV and psychological health may be potentially optimized along with a diet consisting of whole fruits and vegetables. Adding flavonoid-rich berries in the form of a dietary supplement to a whole food diet or taking as a dietary supplement in austere environments may provide an increased health benefit as an antioxidant. A blackcurrant supplement may offer a more concentrated dose of healthful anti-inflammatory properties that may not be obtained solely by relying on whole fruit and vegetable consumption throughout the day. Blackcurrant may help to mitigate proinflammatory and oxidative stress processes and may potentially protect against CV and brain disease.

Nutrition education is another important component to promote the warfighter's nutritional readiness and health. In particular, dietary supplements have become a billion-dollar industry marketing nutritional benefits to both the civilian and the military communities. However, the Food and Drug Administration (FDA) does not regulate

dietary supplements sold in commercial markets as stringently as pharmaceutical drugs. Under the Dietary Supplement Health and Education Act (DSHEA) of 1994, dietary supplements enter the market with less rigorous oversight than pharmaceutical drugs (33; 34). This situation poses challenges to the safety and welfare of consumers because it requires users of dietary supplements to be aware of their ingredients. This challenge presents with potential inadequate consumer knowledge of ingredients and discernment of propriety blends (32; 124). In addition, dietary supplement market strategies that report dietary supplement health and performance may not be research-based. Unlike drugs, where there are FDA research requirements and market exclusivity, such requirements and financial incentives do not exist for nutritional supplements. Frontline healthcare providers can also have a large impact on educating patients about the safety and consumption of dietary supplements. However, they need to be knowledgeable and up to date on the latest information about dietary supplements. More educational training for health care providers may need to be incorporated into their programs of study. Research is needed on various dietary supplements to substantiate their health claims which would justify their use in the military community.

Cell Work to Future Human Study Considerations

In the current research study, carefully designed investigations on blackcurrant extract are planned to include purity testing of the berry extract to be used in the study. If this berry extract is proven effective at reducing oxidative stress when tested in various cell line models, it may prove beneficial to health and warrant translation to the human model and in the military. Although cell culture research is conducted at the basic science level, this research will also provide key insights into the dose-response of berry

flavonoids as well as inform future clinical trials about high and low-performing berry flavonoids. Further investigations may lead to changes in military nutrition lifestyle approaches and may help to increase the operational and health readiness of military personnel. For this reason, blackcurrant extract and other berries will be surveyed and investigated within various cell models of induced oxidative stress to assess the effects on CV and brain health.

Additionally, since CVD statistics continue to rise in the military, this is an optimal time to test novel alternative therapies for improving CV and brain health. In this next section below, the statement of the problem, the purpose of this research, and the hypothesis of the study are presented.

STATEMENT OF THE PROBLEM

Cardiovascular disease is the leading cause of morbidity and mortality in the U.S (13) and impacts approximately 18.1% of active-duty military service members with at least one CVD diagnosis or risk factor (105; 128). Currently, statin therapy is the standard pharmacological treatment used to lower the risk of CVD. However, other effective and safer dietary supplements may decrease the risk of CVD or augment standard pharmacotherapy. There is a lack of nutrition-based interventions specifically for the military population. Since there is frequent use of dietary supplements within the military community, providing research-based dietary supplement (*as a supplement to their diet* with whole fruits and vegetables), that meets their lifestyle demands may serve as an effective solution for promoting cardiovascular and brain health. Evidence suggests that fruits rich in flavonoids possess antioxidant and anti-inflammatory properties that

may improve CVD (28). Notably, flavonoids such as those found in blackcurrant extract, reduce oxidative stress, inflammation, and inhibit platelet aggregation, which are key to promoting CV and brain health (28). However, there is a lack of research studies that have investigated the effects of berry extracts on cells that mediate inflammatory or targets of oxidative stress, namely cardiomyocyte and microglia cell model lines. Therefore, this provides an opportunity to address this research gap and to test a novel nutritional strategy, from a basic sciences approach, which may translate to future clinical trials in the military. The new knowledge gained can then be translated to improve CV and brain health.

PURPOSE OF THE STUDY

The purpose of this study is to investigate the effects of blackcurrant extract on oxidative stress within various cell models (cardiomyocyte and microglia cell model) representative of CV and brain health at a cellular level. The laboratory study was conducted at the USUHS Department of Anatomy, Physiology, and Genetics. The independent variable is defined as blackcurrant extract as a dietary supplement. The dependent variable is defined as oxidative stress.

The objective for pursuing this project is twofold:

- 1) To contribute to our fundamental understanding of the effects of blackcurrant extract as a dietary supplement on oxidative stress within representative cell models of CV and brain health. The results of the study may lead to a proof of concept and refinement approach toward the development of a novel nutritional strategy to promote CV and brain health in the military community.

- 2) To contribute to our fundamental understanding of the key ingredients of berry extracts and their effects on health. This understudied area may offer a viable solution for enhancing health and readiness in military warfighters.

Specific Aims, Research Questions, and Hypothesis

Specific Aim One (Primary)

To determine if blackcurrant extract inhibits the production of induced oxidative stress within various cell models (HL-1 cardiomyocytes and BV-2 microglial cell line).

Research Question One

Does applying **blackcurrant** have a health benefit?

Hypothesis

H0: Blackcurrant extract will not affect the production of induced oxidative stress in the HL-1 and microglial cell lines.

H1: Blackcurrant extract will significantly reduce oxidative stress in HL-1 and microglial cell lines.

Specific Aim Two (Secondary Aim)

To determine if blackcurrant extract ingredient delphinidin 3-rutinoside (D3R) is the superior active compound inhibiting oxidative stress in HL-1 cardiomyocytes and microglial cell lines in comparison to the lower performing active compound cyanidin 3-glucoside (C3G) found in cranberry extract.

Research Question 2

Is there a difference in antioxidant potency between active compounds of delphinidin 3-rutinoside (D3R) and cyanidin 3-glucoside (C3G) tested on HL-1 and microglial cell lines?

Hypotheses

H0: There will be no difference between the blackcurrant extract ingredient delphinidin 3-rutinoside (D3R) as the superior active compound inhibiting oxidative stress in comparison to the active compound cyanidin 3-glucoside (C3G) found in the lower performing cherry extract on HL-1 and microglial cell lines.

H1: There will be a statistically significant difference between the blackcurrant extract ingredient delphinidin 3-rutinoside (D3R) as the superior active compound inhibiting oxidative stress in comparison to the lower performing active compound cyanidin 3-glucoside (C3G) tested on HL-1 and microglial cell lines.

SIGNIFICANCE OF THE STUDY

The warfighter has unique operational demands in facing remote austere environments and high levels of stress that may impact CV and brain health. Strategies to improve warfighter health and consideration of their military lifestyle are crucial towards improving the warfighter's nutritional and health status. Therefore, it is paramount to identify safe alternative therapies that promote CV and brain health in the military community. In that regard, many investigators have focused on flavonoids due to their anti-inflammatory, anti-oxidative, and antiplatelet aggregation properties (98). The proposed research may give rise to a novel proof of concept for an adjunctive therapy using a dietary supplement, blackcurrant extract, to reduce oxidative stress. If this cell

study is proven effective, further research should be conducted in a clinical trial with human subjects to test the relevancy of a berry extract in promoting CV and brain health of warfighters. The outcomes and implications of this research proposal are expected to inform scientists, military leaders, and soldiers on the antioxidant potential of blackcurrant extract for health. In addition, this line of research may also proffer greater interest and focus on the potential of berry flavonoids and their application to the Army's Holistic Health and Fitness model (H2F). Lastly, this research will add to the existing body of science in this area.

The following definitions below will define common terminology that will be used throughout this paper to describe pathophysiological processes, study interventions, and the dietary supplement of blackcurrant extract.

DEFINITION OF TERMS

Atherosclerosis: a vascular inflammatory response that triggers the adhesion of monocytes, leukocytes, and cytokines to the endothelial cell wall. Once lipids invade the endothelium they are oxidized and further attract cytokines and monocytes which differentiate into macrophages that become foam cells. Foam cells are an indicator of fatty streaks, which is a hallmark sign of atherosclerosis. As further inflammation occurs, it can weaken the fibrous cap leading to rupture and eventual thrombus formation and occlusion in the coronary artery (30; 111).

Blackcurrant supplement: a type of berry extract and dietary supplement sourced from Northern Asia and Europe. Blackcurrant, also known as *Ribes nigrum* or *cassis*, which is a woody shrub, is derived from the blackcurrant plant with berries that are rich

in flavonoids. They have anti-oxidant, anti-inflammatory, free radical scavenging activities, and DNA damage repair properties (28).

Cardiomyocyte cells (HL-1): Cells that model cardiac muscle structure and function; this particular cell line is made from transgenic mice atrial cardiac muscle which was induced with an SV40 large T antigen. This particular cardiomyocyte cell line can be serially passaged and has been studied robustly in cardiac structure and cardiac physiology settings (25).

Cardiovascular disease (CVD) or heart disease: can pertain to various heart conditions that involve the heart and blood vessels. Risk factors for CVD include high cholesterol (hypercholesterolemia), high blood pressure (hypertension), smoking, poor diet, obesity, and a sedentary lifestyle. CVD may lead to serious conditions of narrowed or blocked arteries (coronary artery disease) resulting in chest pain, myocardial infarction (injury/damage to the heart muscle from a lack of blood flow and oxygenation), or stroke (26).

Dietary supplement: products that contain at least one dietary ingredient such as vitamins, minerals, amino acids, and their constituents. *It is intended to be a supplement to the diet*, must be consumed by mouth in capsule, tablet, or liquid form, and is labeled as a dietary supplement (33).

Flavonoids: common polyphenols found in most fruits and vegetables, such as blackcurrant, blueberries, green tea, and chocolate. They are water-soluble and composed of anthocyanins which produce purple, blue, and red hues in flowers and fruits. Flavonoids have also been linked to health benefits such as antioxidant, anti-inflammatory, anti-platelet activities as well as lowering blood lipoprotein levels. These

health-promoting benefits have been associated with decreases in heart disease and improved cognitive and psychological health (12).

Inflammation: is the body's immune response to pathogens, irritants, and injury to aid in tissue repair and removal of harmful stimuli. Inflammation influences a cytokine environment through the generation of interleukins, oxidative stress, and monocytes differentiating into macrophages as a host defense strategy. Inflammation is a complex interaction between immune cells, chemoattraction of other inflammatory mediators and cells, and blood vessel responses. Inflammatory processes also initiate atherosclerosis through an accumulation of lipoproteins that are oxidized in the vessel wall. These oxidized lipoproteins lead to the development of foam cells, plaque formation, and vascular dysfunction (39).

Microglia cells: Microglia are the resident phagocytes of the brain. Microglia make up an estimated 12% of cells within the brain. Microglia in their healthiest state is in a rested state; however, when there is a brain injury, trauma, inflammation, or infection, microglia will transform from a rested to an activated microglia posture. Microglia's primary roles are cellular maintenance and host defense (117).

Oxidative stress: an imbalance between reactive oxygen species (ROS)/reactive nitrogen species (RNS) and antioxidant defense mechanisms (64). Oxidative stress is generated when a molecule has an unpaired electron producing ROS or singlet oxygen. Examples of ROS include peroxides, superoxides, hydroxyl radicals, and nitric oxides, all of which can produce harmful effects in the body. ROS are generated through endogenous and exogenous pathways (invasion of pathogens, inflammation, stress, environmental pollution, poor diet, DNA, and myelin sheath damage). They function

physiologically in neutralizing foreign bodies and as signals for inflammatory responses. Overproduction of free radicals can cause chronic diseases such as CVD, cancer, as well as neurodegenerative damage. Increasing antioxidant intake neutralizes free radical production and may be protective against oxidative stress. Antioxidants are molecules that can safely donate electrons to free radicals to terminate the process of oxidative stress leading to chronic conditions (65).

The next section addresses assumptions, limitations, and delimitations of the current phenomenon being studied to provide a comprehensive assessment of the benefits and risks of undertaking this current research.

ASSUMPTIONS

Several assumptions underlie this current phenomenon of investigating the effects of blackcurrant on oxidative stress in cardiomyocyte and microglial cells. The first assumption is that there will be no interference or disruption of cell integrity during routine interventions of cell management. Secondly, the oxidative stress inducer menadione, which is vitamin K3 (involved in physiological clotting factors) is assumed to be similar to other generated oxidative stress signals in the body. Thirdly, the fluorescent dye CellROX™ used to identify oxidative stress in the cell wells will respond to all cell conditions. Fourth, the antioxidant effect found in cell cultures will be similar to human effects. Fifth, there will be no equipment failures during conducting experiments, and there will be minimal investigator fatigue carrying out meticulous cell techniques. Sixth, there will be no data collection errors or system changes that may affect the overall conditions of the cell study.

LIMITATIONS

Several limitations of the current study cannot be controlled by the principal investigator. First, cells are confined within highly controlled experimental conditions, and this does not always translate to human studies. Second, due to limited funding resources, scientific strategies must be prudently thought out for resource management. Third, in a cell-based model system, there is isolation to cell types so there will be no crosstalk communication as in a physiological human system; and this is the rationale for proposing further clinical trials with berries in which blackcurrant extract can be investigated under normal physiological conditions with a multi-organ system.

DELIMITATIONS

The delimitations of this study address the rationale for the chosen investigation, with factors under the control of the investigation, and the boundaries set to narrow the study aim. The current study was selected to develop a better understanding of a particular underlying mechanism of atherosclerosis, which is oxidative stress. Overproduction of oxidative stress is a primary factor initiating further inflammatory processes and leading to many chronic conditions such as CVD. Developing an understanding of oxidative stress at the basic science level using a cell culture model approach may lead to the discovery of safe and alternative nutritional therapies to reduce oxidative stress. Therefore, a study examining the effect of berry flavonoids on oxidative stress may offer key perspectives on specific mechanism action, behavior, and the response of berries. In addition, a comparison of high and low-performing active ingredients found in berry extracts will be tested to identify specific ingredient

effectiveness and to optimize potential therapies. First, cell models were selected as a starting point for understanding the mechanism of oxidative stress. Second, specific cell lines were selected that mimic CV and brain health function; and to test if there is a different response to berry extracts depending on the cell type. Third, the research question and mechanism of action support the rationale and scope of the investigation. Fourth, the methodology of carrying out a cell-based study was chosen as a strategy to provide relevant information about blackcurrant extract toxicity on cell viability, oxidative stress, and the ability to provide a controlled environment for testing and minimizing research biases.

CONCLUSION

In a recent study investigating the CVD rates in the active-duty military community, it was reported that the active-duty population had a higher prevalence rate of less ideal CVD health in comparison to the civilian community (97). This is concerning when one considers the call for the Armed Forces to be ready at a moment's notice to defend the United States of America. Prevention strategies to target and decrease underlying mechanisms of atherosclerosis such as oxidative stress are imperative. Using cell models to investigate berry flavonoids on oxidative stress processes is key for the detection and development of potential novel treatments.

Therefore, pursuing this current investigation may yield a greater benefit of understanding various nutritional strategies to promote CV and brain health at the cellular level. Moreover, an innovative approach with the use of a research-based berry extract may be developed as a remedy to reduce oxidative stress responses. To assist in

illuminating our understanding of mechanisms of action of flavonoids, the next section will provide a literature review of berry flavonoids in cell studies, and supplemental human and animal studies will be included that have also investigated their association with oxidative stress and inflammatory biomarkers.

CHAPTER 2: Literature Review

INTRODUCTION

Optimal CV health is paramount for the operational readiness of today's warfighter; and a dietary supplement with a berry extract may promote CV health as an alternative to pharmacologic interventions. Epidemiological studies have shown that flavonoids found in fruits and vegetables offer CV and neuroprotective benefits (20; 53). An emerging and promising nutritional area of science is the study of flavonoids. Flavonoids are plant-derived bioactive compounds with anti-inflammatory, cardioprotective, neuroprotective, and anti-oxidative properties (37; 58; 106). Flavonoids have subclasses, including anthocyanins, that contribute to the rich color sources of red, purple, and blue naturally characterized in fruits, flowers, and plants (70; 118). Flavonoids also have health-promoting effects. Common foods containing flavonoids are blackcurrants, chocolate, tea, wine, grapes, cherries, cranberries, and blueberries (106). Of particular interest is blackcurrant (*Ribes nigrum*). Blackcurrant is a natural shrub with an anthocyanin-rich berry fruit that is native in northern Europe and Asia (28; 118) and is also currently sold as a dietary supplement. Common ingredients found in blackcurrant include delphinidin-3-glucoside, delphinidin 3-rutinoside, cyanidine-3-glucoside, and cyanidine-3-rutinoside which are associated with inhibiting platelet aggregation, possessing radical scavenging and vascular relaxing activities, and inhibiting cholesterol uptake (28; 47; 108; 118). These properties are reported to down-regulate oxidative stress and inflammatory processes, decrease blood pressure, improve the lipid profile, increase endothelial function, and may improve brain health (19; 106). A novel way to investigate the effectiveness of blackcurrant on CV and brain health is to study a mechanism for

atherogenesis namely, oxidative stress. The primary focus of this study was on flavonoids and their effects on CV and brain health. This review will assess the potential benefits of flavonoids within both *in vitro* and *in vivo* models. The effects of berry flavonoids in various cell line models will be reviewed in the context of their response to oxidative stress. Specifically, it will be important to understand at the cellular level how berry flavonoids may be able to play a role in reducing oxidative stress, which can lead to chronic diseases such as atherosclerosis. Using cell line models as an initial step for CV and brain health investigations of oxidative stress may inform further clinical trials and provide a rationale for dietary supplementation.

In particular, little research has been conducted using berry flavonoids on cell lines of CV origin including cardiomyocytes; however, more studies are being done utilizing BV-2 cell lines of microglial origin. Additionally, both human and animal studies will be included in this literature review to provide the evidence to support berry flavonoids and their beneficial effects on CV and brain health. Both human and animal studies provide relevant literature regarding the bioavailability of flavonoids and their effects on biomarkers of health status such as oxidative stress and inflammatory biomarkers, and psychological health.

Presently, there are significant gaps in the literature on the role of dietary supplements on CV and brain health in the military population. Therefore, the purpose of this chapter is to critically review the relevant literature in three model systems. The literature will be reviewed on the effects of berry flavonoids on cell line models, in human subjects, as well as animals with CV and brain health/mood. Studies investigating mechanisms of oxidative stress and inflammation will also be presented. Classic and

current studies were selected based on their relevance to berry flavonoids, oxidative stress, CV and brain health, procedural rigor, and overall study quality. Classic studies were defined as research articles before 2010 or foundational studies within the berry flavonoid and cell model lines of interest related to the current research investigation.

REVIEW OF RESEARCH

Studies are organized beginning with *in vitro* (cell studies) and continuing with *in vivo* (human and animal studies) and thematically by flavonoid category and their effects on CV and brain health/mood, and mechanisms of oxidative stress, and inflammation.

SEARCH DESCRIPTION FOR *IN VITRO* STUDIES (CELL STUDIES)

The following databases were searched on April 15, 2021, for relevant articles in PubMed, CINAHL, Web of Science, and EMBASE. The literature search included combinations of keywords and appropriate subject headings for each database that retrieved studies focused on the use of cell models in the investigations with berry flavonoids and their effects on oxidative stress in cardiomyocyte and microglial cell lines. Results were limited to English only. Human and animal studies as well as cell culture studies were included in the search. The search strategy identified a total of 89 articles (after the removal of 39 duplicate articles that appeared in the search engine more than once). A total of 50 articles were reviewed and the most relevant studies were included within the literature review.

To ensure each concept was fully represented for berry flavonoids, terms such as “bilberry,” “cherry,” “blueberry,” “lingonberry,” “elderberry,” “strawberry,” and “cranberry” were used. For the concept of cell models, the following terms were

included: “microglia,” “bv-2,” “cardiac muscle cell,” “rat-atrial-cell,” “cardiac myocyte,” “oxidative stress,” or “inflammation.”

HALLMARK STUDIES ON FLAVONOIDS AND CELL TYPES: CARDIAC AND MICROGLIAL CELLS

In a classic study utilizing cells of cardiac origin, H9c2 rat cardiomyoblasts cells, the effects of lingonberry extracts rich in flavonoids on hydrogen peroxide-induced apoptosis or cell death were investigated (57). This was reportedly the first study conducted using human bioavailability doses in an *in vitro* model. Wild lingonberries were picked fresh and immediately frozen at -20 °C. Berries were lyophilized (freeze-dried) and made into a fine powder and diluted for cell culture. Fluorimetrically, the oxygen radical absorbance capacity (ORAC) was determined. Six-well plates were used for seeding (plating) of cells 48 hours before the time when experiments were conducted. Hydrogen peroxide (600 uM) was used to induce apoptosis for two hours after pre-treatment of lingonberry extract for 24 hours at three concentrations (1:1000, 1:2000, and 1:500). Pre-treatment of three lingonberry anthocyanins (cyanidin 3-galactoside (C-3-Gal) (20 nmol L), cyanidin-3-glucoside (C-3-Glu) (20 nmol L), and cyanidin-3-arabinoside (C-3-Ara) (22 nmol) were applied to the H9c2 rat cardiomyoblast cells. Phosphate-buffered saline (PBS) was used as a cell wash. Trypsin-EDTA (0.25%) was applied as an agent to gather and collect cells; cells were centrifuged at 300 revolutions per minute (rpm) for five minutes. Additionally, cells were counted using a Countess Cell Counter to detect cardiomyocytes, and flow cytometry was used to assess their characteristics. Fluorescence microscopy was also used for chromatin condensation detection of apoptotic and non-apoptotic nuclei using image software. This is important

for assessing the effect of hydrogen peroxide on cells and identifying whether lingonberry extract induced changes in oxidative stress in the cardiomyocytes.

The findings of this study demonstrated that lingonberry extract had a high antioxidant capacity *in vitro*. Interestingly, when comparing the antioxidant capacity of lingonberry extract (*dry weight* per 100g) to *fresh weight (after harvest* per 100g), lingonberry extract's dry weight demonstrated higher antioxidant capacity. Lingonberry extract dry weight showed higher antioxidant potential in phenolic content, radical scavenging potential, ferric reducing antioxidant power, and oxygen radical absorbance capacity using four different antioxidant assays.

Moreover, all three anthocyanins found in lingonberry including C-3-Gal, C-3-Glu, and C-3-Ara were found to be protective against hydrogen-peroxide-induced oxidative stress in rat cardiomyoblasts (57). Apoptosis induced by hydrogen peroxide was also demonstrated after a significant increase in caspase-3 activity, which is an indicator of cell apoptosis. When the percentage of apoptotic cell totals were compared between the hydrogen peroxide and untreated groups versus combination hydrogen peroxide and berry extracts groups, the results showed that the percentage of apoptotic cells was significantly lower ($p < 0.05$) in the combination of hydrogen peroxide and berry extract groups in comparison to the hydrogen peroxide only and untreated groups. This suggests that lingonberry extracts have a cardioprotective effect on rat cardiomyoblast cells in the presence of hydrogen peroxide-induced oxidative stress. Interestingly, at concentrations of lingonberry (1:500) and with the addition of hydrogen peroxide, the greatest decrease in the percentage of apoptotic cells was observed in comparison to all the other combinations of berry extract and hydrogen peroxide groups.

In the statistical analyses of the results, analysis of variance (ANOVA) was appropriately used to compare means between groups, and Tukey's test was used to compare significantly different means. In conclusion, this study demonstrated that lingonberry extract rich in anthocyanins have high antioxidant and radical scavenging activity; and provide cardioprotection from hydrogen peroxide-induced oxidative stress in cardiomyoblast cells. As a noted weakness of this study, only a single berry extract was used with the active compound ingredients as a comparison. However, this investigation was notably the first study to use a human bioavailable dose or concentration of intact anthocyanins of berry extracts in a cell model.

In another hallmark study, American ginseng berry (AGBE) was investigated due to its enhanced antioxidant potential (92). Before this study, researchers were unsure of this plant's phenolic content. Therefore, AGBE was purchased and lyophilized after seeding (plating of cells), and dissolved in a culture medium, and filtered before its use. The sample used in this study were cultured chick embryo ventricular cardiomyocytes from 10-day embryos. The cardiomyocyte cultures were separated, minced, and trypsin (0.025%) was used for cell division of adherent cells. The cell purity process included the collection of cell suspension and centrifugation, and then the remaining cell pellets were resuspended in a culture medium. After this process, cell viability was measured by trypan blue which detects dead cells. Cell viability was greater than 95%. Cells were then plated and incubated with carbon dioxide (CO₂) (5%) at 37°C. Oxidative stress was induced with hydrogen peroxide. As for cell death measurement, propidium iodide, a fluorescent stain for chromosomes that is excluded from healthy cells, was used to assess cell viability after hydrogen peroxide exposure.

The cardiomyocytes were pretreated with AGBE at dose ranges of 0.5, 1.0, and 2.5 mg/ml at time points 24, 48, and 72 hours. Individualized active ingredients, caffeic acid and chlorogenic acid found in AGBE were procured from a reputable chemical company noted for their quality pure ingredients. These ingredients were individually analyzed by high-performance liquid chromatography (HPLC); a technique that entails identifying the content, testing purity, and quantification of a certain compound. Cells were visualized with fluorescence and quantified using imaging software.

Dichlorofluorescein diacetate (DCFH/DA) was used to measure oxidative stress by a fluorescent product. Measures were taken from fluorescence at 60 minutes. In preparation for the assay, cardiomyocytes were washed with a balanced salt solution (BSS) twice for removal of the remaining AGBE in the wells. As for findings, cardiomyocytes that were pretreated with AGBE at dose ranges of 0.5, 1.0, and 2.5 mg/ml at time points 24, 48, and 72 hours had a significant reduction in intracellular oxidative stress with a significant dose time response ($p < 0.005$). Whereas all dose responses indicated a significant reduction in DCF fluorescence in the cardiomyocytes after hydrogen peroxide exposure ($p < 0.005$), the 72 hours of pretreatment with AGBE in comparison to the 24 hours of pretreatment showed the greatest decrease in DCF fluorescence. This suggests that AGBE used over longer periods may significantly and positively improve cell survival. Additionally, identifying the effects of caffeic acid and chlorogenic acid treatment on hydrogen peroxide-induced oxidation demonstrated that these compounds were able to significantly reduce ($p < 0.001$) induced oxidative stress at the 5 μM and 15 μM dose range. Only caffeic acid at the 2.5 μM concentration significantly reduced oxidative stress ($p < 0.001$) in comparison to the chlorogenic acid at a similar dose. A strength of this study

is that it was the first study to find that AGBE has polyphenolic content. This study also showed that the polyphenols were tested with induced oxidative stress. The results demonstrated that AGBE has an antioxidant protective effect in cardiomyocytes.

The effects of berry flavonoids on brain health were investigated in microglial cells. Microglial cells play a crucial role in the immune response of the brain by acting as a host defense (117). Microglial cells have macrophage properties and can produce oxidative and inflammatory responses to injury, infection, and aging progression (7; 117). Therefore, researchers have become increasingly engaged and interested in microglial cell responses and finding solutions that may enhance brain health. When microglial cells are overly excited, it can interfere with neuronal networking and increase neuroinflammation leading to cognitive/mood decline, depression, and neurodegenerative diseases (7; 134). For this reason, microglial cell models are a good foundational model to investigate properties of inflammation and oxidative stress as indicators of brain health. Additionally, nutritional interventions, such as berry flavonoids, can be tested in microglial cells to investigate their role in brain health. To investigate this further and to provide evidence for testing flavonoids in this model, a study will be presented on the effects of berry flavonoids on oxidative stress and inflammation utilizing the microglial cell model in the following section.

Since microglial cells are linked to important processes of the brain and immune response regulation, researchers investigated tart cherries rich in flavonoids, and their effect on oxidative and inflammatory mediators in microglial cells (130). HAPI rat microglial cells were used in this experiment as a sample. Cells were passaged in 12-well plates and stored at 37 °C in a humidified incubator under 5 % CO₂ and bicarbonate

buffer. Montmorency tart cherry powder was diluted with media or control media and pretreated in cells at concentration doses of 0.062, 0.125, 0.250, 0.500, and 1.000 mg/ml for one, two, and four hours. Next, the cells media was aspirated after pretreatment, and lipopolysaccharide (LPS) was applied to cells to induce inflammation and oxidative stress. Cell viability was captured using a live/dead viability cytotoxic kit. Calcein AM (an esterase stain incorporated into healthy cells) was used to label live cells with green fluorescence and Ethidium homodimer-1, (a high-affinity nucleic acid stain normally excluded by healthy cells) was used to measure dead cells with fluorescence red. Nitrite was assessed to detect nitrogen production from the cells as a measure of inducible nitric oxide synthase (iNOS) which is a signaling molecule of nitric oxide. The western blot test was used to identify proteins such as COX-2 and iNOS. Additionally, Tumor Necrosis Factor α (TNF α) was assessed as an inflammatory cytokine using a quantification method called enzyme-linked immunosorbent assay (ELISA) which can detect antibodies *in vitro* and *in vivo*. Data analysis included the use of ANOVA and Tukey's test for determining the differences and significance between the tart cherry dose and time effects. The results indicated that the higher concentration of tart cherry extract (1.000 mg/ml) caused cell toxicity and death. Doses at 0.500 mg/ml and lower concentrations were more favorable at testing the berry extract protective properties in microglial cells. Longer duration times of tart cherry pre-treatment of two and four hours also caused slightly decreased cell viability in comparison to the control cells. The pre-treatment of tart cherry was effective in weakening LPS induced nitric oxide production in comparison to the control; significance was reached at dose responses of 0.250 and 0.500 mg/ml at one hour ($p < 0.05$). Also, doses of 0.125, 0.250, and 0.500 mg/ml with the

pre-treatment tart cherry significantly reduced NO production ($p<0.05$) in comparison to the control group at both the two-and four-hour time frames. As for iNOS, there was a significant increase in NO production ($p<0.05$) after LPS application in all groups in comparison to the control. Pre-treatment of tart cherries significantly reduced TNF α in a dose-response manner; LPS was significantly induced ($p<0.05$) and stimulated TNF α in all groups in comparison to the control group. Interestingly, lower doses of tart cherry significantly decreased COX-2 inflammatory mediators. These findings demonstrate that rat HAPI microglial cells may be a good model for testing the effects of oxidative stress and inflammatory mediators in the presence of berry flavonoids. Loading up cells as a pre-treatment with tart cherry in this study demonstrated a protective effect against oxidative stress and inflammation in the microglial cells. Tart cherry may also be a potent antioxidant and anti-inflammatory fruit extract; however, its effects may depend on dose concentration, duration, and pathway targeted. In conclusion, this study added to the literature an important investigation utilizing tart cherry, a flavonoid-rich berry to demonstrate the oxidative and inflammatory pathways affected in the HAPI rat microglial model. It also showed that the effects of tart cherry are highly dependent upon cell viability. Prolonged exposure of berry extracts in cells and at higher concentrations may be cytotoxic rather than protective. In the present proposal, the dose-response of berry extracts on cardiomyocytes and microglia cells were examined with a menadione agent (K3) that induces oxidative stress as the primary focus. Cell viability was also assessed in the presence of the berry extracts and induced oxidative stress to determine if the berry extracts have CV and neuroprotective properties.

In conclusion, the studies presented on the effects of berry flavonoids in cardiac and microglial cell lines demonstrate the value of these nutrients in ameliorating oxidative stress and inflammation. These beneficial properties may contribute to overall CV and brain health benefits over time. Both the lingonberry and AGBE studies used hydrogen peroxide as an inducer of oxidative stress. However, the tart cherry study used LPS as a stimulator for oxidative stress and inflammation, demonstrating that oxidative stress can be induced with multiple reagents for cell culture applications. In addition, berries are comprised of different chemical compounds or flavonoids; they have different dose responses, and cell viability toxicities. However, all the berry extracts demonstrated beneficial properties that can protect cells through their antioxidant and anti-inflammatory properties. It is important to note that there may also be cytotoxic effects at a concentration of 1.000 mg/dl or higher in certain berry extracts, such as tart cherry, as previously shown (57; 92; 130). However, at lower doses of tart cherry (0.250 and 0.500 mg/ml), there is a cell-protective effect (130). In the case of some berry flavonoids, there was also a benefit to longer exposure time (72 hours) such as using AGBE as a pretreatment in comparison to using 24-hour pretreatment; the study showed that it improved the oxidative stress response (92). Overall, these cell studies show that there are potential antioxidant benefits to using berry extracts or flavonoids in a time-dependent and dose-response manner. Additionally, these investigations suggest that the berry-extract-dominant profile strains or key active compounds should be studied further to determine their effects as free radical scavengers. This is essential to understanding how berry extracts may act as free radical scavengers by interfering with the oxidative stress process through neutralizing free radicals in the body that can cause lipid

peroxidation, as well as damage DNA and proteins (80). If free radicals are left unchecked and remain chronically overabundant in the body, this may lead to certain chronic diseases such as atherosclerosis, depression, and cancer over time (80).

In the next section, the effects of berry flavonoids in both human and animal studies are reviewed in relation to bioavailability after consumption and their role on CV and psychological health.

SEARCH DESCRIPTION FOR IN VIVO STUDIES (HUMAN AND ANIMAL STUDIES)

The following databases were searched on January 21, 2020, for relevant articles in PubMed, CINAHL, and EMBASE. The literature search included combinations of keywords and appropriate subject headings for each database that retrieved articles discussing the use of blackcurrant and its impact on reducing oxidative stress and inflammation as biomarkers in human and animal studies. Results were limited to the English language. The search strategy identified a total of 246 articles (after the removal of 226 duplicate articles that appeared in the search engine more than once). A total of 20 articles were reviewed and the most relevant studies were included in the literature review.

To ensure each concept was fully represented for blackcurrant, terms such as “*Ribes nigrum*,” “*ribes americanum*,” “black currant,” “blackcurrant,” and “*grossulariaceae*” were used. For the concept of biomarkers, the following terms were included: “lipids,” “cholesterol,” “low density lipoprotein,” “biomarkers,” “fatty acids,” “omega-3,” heart, “oxidative stress,” “high-density lipoprotein,” “blood flow,” “biological marker,” or “arterial stiffness,” and “lipid panel.”

After assessing the relationship between biomarkers and CVD is addressed, atherosclerosis as a theoretical framework will be explored in connection to blackcurrant flavonoid and its effect on inflammation, oxidative stress, and mood.

THEORETICAL OR CONCEPTUAL FRAMEWORK

Atherosclerosis is triggered by vascular injury, and the resultant inflammatory response causes macrophage and monocyte recruitment, adhesion of platelets, oxidation of lipoproteins that further the pro-inflammatory cascades, and plaque formation. These responses eventually lead to vascular dysfunction (145). Epidemiological studies suggest that diets rich in fruit and vegetables reduce the incidence of CVD (67; 94; 100). Favorable nutrient properties are thought to be related to flavonoid content, which may protect against CVD (118). The darker the fruit, the higher the flavonoid content (63). Investigators have linked higher flavonoid intake to decreased rates of CVD (116). Studies have found that flavonoid-rich foods such as berries, chocolate, cocoa, red wine, apples, and cherries improve endothelial function, decrease blood pressure, reduce oxidative stress, and improve cerebral blood flow (36; 116). Of particular interest are blackcurrants because of their high level of flavonoids known as anthocyanins (101). Anthocyanins have also been shown to lower oxidative stress, inflammatory markers, and blood pressure (38; 55; 63).

HALLMARK STUDIES ON FLAVONOIDS: FRUITS, VEGETABLES, AND CHOCOLATE

In the last decade, the health effects and cardioprotective potential of fruits and vegetables have been of great interest. Epidemiological studies have demonstrated a positive relationship between flavonoid intake and CV health. For example, the Zutphen Elderly Study, a classic longitudinal study, demonstrated a link between coronary heart disease (CHD) and antioxidants (53). This study did not have a theoretical framework; however, a scientific background was provided on flavonoids. This study originated in 1960 in the Netherlands with a cohort of 878 men. A follow-up study was conducted in 1985 with 555 men from the original cohort who were alive and invited to participate in the study. The target sample for this study was 1266 men. In 1985, 939 men entered the study; however, only 805 participants had complete information on diet and risk factors. Inclusion criteria were men aged 65-84 years old and myocardial infarction (MI) with severe chest pain sustained for over 20 minutes with electrocardiogram changes such as major Q wave and T wave findings by practitioner's documentation. Exclusion criteria were not stated. Procedures used venous blood samples (fasting status unknown) to measure cholesterol and health status levels as related to blood pressure and anthropometrics such as height and weight. Physical exams and dietary intake were assessed. Dieticians estimated the quantity of food consumption in a typical week or by portable scale if necessary. Mean flavonoid levels were calculated based on the sum of antioxidants including quercetin, kaempferol, luteolin, etc., using dietary intake data. The men were stratified into tertile groups by flavonoid intake levels: 0-190 mg/daily (low flavonoid intake), 191-299 mg/daily (moderate flavonoid intake), and >299 mg/daily (high flavonoid intake). One-way ANOVA and Spearman Rank Correlation Coefficients (SAS) were used for statistical analysis of baseline tertile differences of dietary and

supplemental intake. The Chi-square test was used for probability trends. The results indicated an increase in vitamin C (calculated by a trained dietician for usual food intake), which the investigators viewed as indicative of flavonoid consumption. All-cause mortality rates were decreased with increasing flavonoid intake; however, the significance level was borderline after adjusting for age, diet, and risk factors ($p = 0.084$). When relative risk was adjusted for age and diet alone amongst all flavonoid intake groups, there was an increase in significance level for all-cause mortality ($p = 0.039$). As for coronary heart disease mortality rates, there was a borderline decrease from the lowest to the highest tea flavonoid intake group ($p = 0.081$). When the relative risk was adjusted for age, diet, and risk factors there was an increased significance in coronary heart disease mortality rates amongst all the tea flavonoid intake groups ($p = 0.033$). Overall, increased flavonoid intake was associated with decreased CHD mortality. Interestingly, this population had a high intake of the flavonoid quercetin, commonly found in tea. In addition, this study demonstrated an important relationship between high flavonoid intake and decreased mortality rates of CHD. However, a limitation of the study was that the target population of 1266 for the study was not reached due to participants being lost to follow-up due to various reasons such as natural causes and individuals moving (53). The 1985 cohort ended up with a total of 939 entering the study, however, 61 individuals did not have complete data. Therefore, this left only 805 individuals with complete data for the study. In conclusion, this important study highlights the benefits of a high flavonoid intake diet and its properties of anti-oxidation and anti-inflammation.

Another flavonoid-rich source that demonstrates promising protective CV health benefits is dark chocolate. This first-ever study investigated the effects of low-flavonoid

and high-flavonoid dark chocolate on endothelial function (36). The sample size included 21 healthy male and female subjects ages 21–55 years old. The study design was a randomized, double-blind, placebo-controlled investigation. Participants were selected and randomized to consume low-flavonoid (trace) or high (46 g) flavonoid chocolate bars daily for two weeks. The high-flavonoid group received chocolate bars that contained cocoa flavonoids, procyanidin, and epicatechin, which were absent in the low-flavonoid group. Procedures included individual documentation of three-day food records, participants were to consume chocolate bars after lunch and before dinner daily, and to consume a chocolate bar two hours before fasting blood draws. Subjects were instructed not to consume flavonoid-rich foods and beverages before visits and to discontinue any other dietary supplement during the two-week study time frame. High compliance was achieved among participants as monitored with a log and blood epicatechin levels. Validated food frequency questionnaires were completed by participants initially for baseline dietary assessment. Fasting blood samples drawn at baseline and post-study were evaluated for biomarker lipid panels, epicatechin levels, Oxygen Radical Absorbance Capacity (ORAC), and plasma 8-isoprostanes levels (indicative of oxidative stress). High reliability and validity were achieved through proper laboratory handling and storage. Blood pressure and brachial artery flow-mediated dilation (FMD) were obtained for further objective measurements. Results after a two-week intervention showed that the high-flavonoid group had increased significance for mean measures of increased FMD and epicatechin levels compared to the low-flavonoid group ($p = 0.024$). Other laboratory measurements such as lipid panels, oxidized LDL, ORAC, and 8-isoprostanes levels did not differ as a function of the intervention, but this may have been due to the limited two-

week time frame. In conclusion, this landmark investigation suggests that increased epicatechin levels from chocolate consumption were associated with improvement in endothelial function and blood flow, which may indicate enhanced NO production.

EFFECTS OF GRAPES, CHERRIES, AND POMEGRANATE JUICE ON BIOMARKERS

Following epidemiological studies that focused more on the population and dietary intake of flavonoids (20; 53; 96), a team of scientists shifted their attention to more individualized flavonoids and their effects on patients with mild hyperlipidemia and elevated lipoprotein biomarkers (115). The study was designed to determine the effects of red grape seed extract (RGSE) in hospitalized mild hyperlipidemic individuals. This was a randomized, double-blind, placebo-controlled study with an eight-week crossover design. The experimental group was given a capsule with 100 mg of RGSE, which by analysis was found to contain about 95% proanthocyanins and 80% polyphenolic compounds. The placebo group was given corn starch in capsule form. To prevent extraneous variables, both groups were instructed not to change their diet or lifestyle, and not to consume any grape substances to prevent extraneous variables. The sample size included 52 individuals; however, 10 individuals dropped out of the study due to non-compliance or lack of follow-up during the washout phase. The average age of individuals in the study was 48 years. Inclusion criteria were ages 21–64 years, triglyceride levels > 150 mg/dL, and total cholesterol levels > 200 mg/dL. Individuals who were currently taking lipid-lowering medications; had severe hyperlipidemia > 300 mg/L, chronic kidney disease, heart failure; or reported drinking alcohol or smoking cigarettes were excluded. The design selected was appropriate with no internal validity threats, however, there were external validity threats with the 10 individuals who dropped

out that could have affected study generalizability. Endpoints used for the study were blood levels of Ox-LDL, triglycerides (TG), total cholesterol, LDL, high-density lipoprotein cholesterol (HDL), and very low-density lipoprotein cholesterol (VLDL-C). Additional measures included blood pressure, body weight, body mass index, and dietary intake from food frequency questionnaires. The overall findings of the study showed that individuals in the RGSE group had significantly lower blood cholesterol, LDL-C, and Ox-LDL levels compared to pre-levels. The placebo group also changed significantly with increased cholesterol ($p=0.043$) and Ox-LDL($p=0.004$) as compared to the pre-levels. Limitations of this study included the inability to recruit the target sample size of 52 patients, which may have affected generalization. RGSE nutritional composition was not provided in the report, and this may have an impact on individual lipid profiles.

Another study examined the properties of tart cherry juice on inflammatory and oxidative stress biomarkers (21). The study sample included 37 participants (17 men and 20 women) ages 65–80 years old. The study design was a randomized-controlled-trial parallel study conducted over 12-weeks. The subjects were randomized to receive either 480 ml of the experimental Montmorency tart cherry drink daily for 12-weeks or the control drink daily for 12-weeks. The control drink contained unsweetened black cherry Kool-Aid and sugar to match the color of the tart cherry drink. Measurements collected included blood pressure, height, weight (digital scale), and body mass index (BMI, kg/m^2). Individuals who consumed fewer than five servings of fruits and vegetables daily determined by a food record were eligible to participate. Individuals who were on medications for CV or cognitive decline or had a history of heart disease, stroke, psychiatric disorders, or other chronic conditions were excluded. The study provided

reliability and validity powered at 80% with a sample size of 36 and an alpha of 0.05. Results showed no changes in body weight in either group after the 12 weeks intervention. Biomarker changes included a significant decrease in CRP levels, an indicator of decreased inflammation, in the tart cherry group compared to the control group ($p=0.03$). Concentrations of 8-oxoguanine DNA glycosylase (OGG1), an indicator for gene coding and DNA repair, were significantly increased in the tart cherry group ($p<0.0001$). Lastly, malondialdehyde (MDA) levels, a biomarker for oxidative stress, were significantly decreased in the tart cherry group compared to the placebo group ($p=0.01$) after the 12-week intervention. This study was the longest tart cherry intervention study conducted to date, and although some interesting findings emerged, the study lacked generalization as the sample consisted of an older adult population. More studies would be needed to show a consistent effect of tart cherry juice on inflammatory and oxidative stress biomarkers in a younger population.

OXIDATIVE STRESS AND BIOMARKERS

In another study with similar flavonoid composition as tart cherry, the effects of high blackcurrant (81.5 mg/100 ml) and low blackcurrant (27.3 mg/100 ml) juice on oxidative stress were examined in a healthy population with low baseline flavonoid intake. This study assessed the benefits of blackcurrant juice on flow-mediated dilation (FMD) in a double-blind, placebo-controlled, parallel study design over six weeks (68). The sample size included 66 healthy participants (average age 53 years old) that were randomized to three arms of the study (placebo group, low blackcurrant group, or high blackcurrant group). The investigational treatments were administered in juice form. The low blackcurrant group received 27.3 mg/100 ml in polyphenols, the high blackcurrant

group received 81.5 mg/100 ml in polyphenols, and the placebo group received only flavored water. Participants were recruited from the general population in Scotland via newspaper, poster advertisements, and lay press. Dietary assessment included completion of a three-day fruit and vegetable dietary record at the initial visit. Individuals were asked to fast 12 hours before, and the initial visit when measurements of FMD were taken in a temperature-controlled room after 15 minutes of rest at the initial visit to assess endothelial function. Three consecutive FMD measurements were taken, and the last two measurements were used for analysis. Participants were instructed to drink 250 ml of the respective juice four times a day for six weeks. Measurements taken at baseline were taken repeated at a six-week follow-up visit. Compliance for intake of fruit and vegetables was measured by using a Five A Day Community Evaluation Tool (FACET) tool at three and five weeks. Results indicated that participants in the low and high blackcurrant supplement groups had significant increases in plasma vitamin C levels as compared to the placebo group. F2-isoprostanes plasma levels, an indicator of oxidative stress, were also significantly lower in the high blackcurrant group in comparison to baseline levels after 6 weeks. The second major finding was a significant improvement in FMD in the high blackcurrant group as compared to the placebo and low blackcurrant groups. One strength of this study was the high compliance rates for participants. However, a limitation of the investigation was the study duration of only six weeks. Different effects of the supplements may occur with longer-term clinical trials. In conclusion, the study findings demonstrate that blackcurrant supplements improved FMD which may be suggestive of increased nitric oxide production and decreased oxidative

stress. Overall, blackcurrant supplement consumption at a high level for six weeks improved endothelial function and had an antioxidant effect.

EFFECTS OF POMEGRANATE JUICE ON INFLAMMATORY AND OXIDATIVE STRESS BIOMARKERS

In a study published in 2016, pomegranate juice (PJ) which is rich in flavonoids was used as a dietary supplement drink in a five-week double-blind, parallel, randomized control trial (43). The purpose of the study was to assess the effects of PJ on inflammatory and oxidative stress markers in a sample of 31 endurance athletes in Spain. The participants were randomized into three groups including pomegranate juice (PJ), pomegranate juice diluted (PJD), and control (C). Athletes were recruited from surrounding sports clubs. Inclusion criteria were healthy adult men ages 33-37 years old with the ability to perform endurance-based exercise training sessions. Exclusion criteria were consumption of antioxidants or anti-inflammatory supplements, chronic diseases, and alcohol intake. Participants were given customized dietary plans. Baseline and end-of-study blood samples were collected to assess the role of pomegranate on oxidative stress. Measurements were obtained at the initial start of the study and day 22 or the end of the five-week study. Measurements included height, weight, BMI, health status biomarkers. The focus for this study included the results for the following blood tests: glucose, HDL levels, potassium, lactate, and ferritin, as well as oxidative and inflammatory biomarkers. Oxidative biomarkers included plasma protein carbonyl and malondialdehyde (MDA). Inflammatory biomarkers included CRP and sE-selectin. Results showed that lactate levels significantly increased (potentially resulting from high-intensity exercise) in the PJ group ($p < 0.01$) from baseline to day 22 compared to the

control group. This may contribute to an understanding of fatigue perception. Ferritin levels did not differ significantly between the control and PJD groups at baseline, but levels had decreased significantly in the PJ group ($p<0.05$) at day 22. As for potassium levels, both the control and PJD groups were non-significant; however, the PJ group did show significance with increased potassium levels ($p<0.05$) in comparison to the control and PJD groups from baseline to day 22. HDL levels were significantly higher in only the PJD group ($p<0.05$) compared to the other groups. There were no significant differences in biomarkers or lipid panel parameters after PJ consumption. Oxidative stress biomarkers, plasma protein carbonyl, and MDA results indicated there was a significant increase from baseline to day 22 in oxidative stress for the control groups that were not supplemented with PJ ($p<0.01$). There was also a significant decrease in MDA levels in the PJ and PJD groups ($p<0.01$). There were no significant changes reported for inflammatory biomarkers, CRP or sE-selectin, during the study.

Limitations of the study included the loss of 11 participants due to study violations. No description was provided on the specific exercise endurance intervention of the participants. However, participants were asked to make their exercise session over an hour in length and to participate more than three days out of the week in an endurance exercise regime. In conclusion, the study demonstrated that flavonoid rich PJ may have a beneficial effect by reducing oxidative stress during exercise endurance.

In summary, previous studies have shown that flavonoid-rich fruits, chocolate, and flavonoid-enriched juices have significant effects on vascular health and biomarkers related to mechanisms associated with atherosclerosis (inflammation and oxidative stress). However, limitations to these studies included low participant numbers under 100

individuals; but significance in berry flavonoid effects were reached despite the recruitment numbers ranging from 21-52 subjects in the studies described. In addition, there were 10 or more participants who dropped out of two of the studies for various reasons. In contrast, there is more control for the investigator in cell culture studies. In the proposed cell study, cells are readily available to test berry extracts with a robust population of single cells. Additionally, experiments can be conducted at a more rapid pace in cell cultures as a way to understand the underlying mechanisms and the effects of berry extracts on outcome measures. However, interventions may require two to 12-weeks or more in human studies to determine the effects of berry extracts on outcome measures such as oxidative stress.

In the next section, the effects of various flavonoids in animals and additional human studies are addressed. These findings in the animal model add to our scientific knowledge obtained from human studies.

EFFECTS OF BLACKCURRANT SUPPLEMENTATION IN VIVO

In one key study, Wistar rats were fed either an unprocessed or processed blackcurrant preparation to investigate the effects of a polyphenol-rich supplemented diet on biomarkers of oxidative stress and lipids (61). The sample included 48 Wistar male rats aged seven weeks. The animals were randomized into six groups of eight rats each placed on a four-week dietary plan. The three main dietary groups included standard animal feed, 5.98 mg/g of polyphenols (unprocessed blackcurrant), or 2.53 mg/g of polyphenols (processed blackcurrant). The remaining groups received diets that were starch or fructose based that equaled 3.59 kcal/g. Reliable and valid measures were used such as standard laboratory detection kits for blood serum biomarkers and two-way

ANOVA analyses were used to identify the effects of blackcurrant supplement products (unprocessed and processed forms) on oxidative stress biomarkers and lipid profiles. The results indicated that the biomarker thiobarbituric acid reactive substances (TBARS), a byproduct of lipid peroxidation, was significantly reduced in rats fed a fructose high diet and supplemented with unprocessed blackcurrant (BC) pomace compared to the control group. This suggests that oxidative stress was decreased in the BC group. Also, consumption of BC pomace (processed and unprocessed) significantly decreased free fatty acids (FFA) and triglyceride (TG) levels. Interestingly, only the blackcurrant unprocessed pomace group significantly decreased total blood cholesterol levels. A limitation of the study was the lack of measurement of inflammatory biomarkers which would have provided an assessment of the potential anti-inflammatory effects of BC. However, before results can be generalized, human studies must be done using different BC pomace concentrations.

In conclusion, consumption of processed and unprocessed BC pomace improved blood lipid profiles and were associated with increased antioxidant capacity and decreased lipid peroxidation in rats. These findings suggest that short-term dietary supplementation of blackcurrant has a lipid-lowering effect and reduces oxidative stress in the animal model. Therefore, this study revealed that blackcurrant has important effects and mechanisms that may be inhibitory to atherogenic processes.

EFFECTS OF FREEZE-DRIED BILBERRY ON EXERCISE TOLERANCE AND BIOMARKERS FOR CORONARY ARTERY DISEASE

Bilberries, a subclass of berries containing flavonoids, may offer cardioprotective health benefits by affecting platelet function, lipid profiles, and inflammatory processes (5). One recent study tested a freeze-dried bilberry powder supplement on participants who had recently experienced a myocardial infarction during an eight-week prospective, open-label, randomized clinical control trial. The sample included 60 participants (42 males and 18 females) with an average age of 68 years and a percutaneous coronary intervention (stent placed to open up blocked blood vessels). Primary outcomes were measures of inflammatory biomarkers and the results of a six-minute walking test (6MWT). Participants were instructed to remain on their current statin therapy throughout the study. Inclusion criteria included ST-segment elevation myocardial infarction (STEMI) or non-ST-elevation myocardial infarction (NSTEMI). Exclusion criteria included participants who received urgent coronary artery bypass grafting, age less than 18 years, daily bilberry intake, and anyone currently enrolled in the BEARSMART trial. Baseline and eight-week study assessments with the collection of blood samples were performed. Subjects were randomized either to the bilberry supplement or no dietary intervention group. Instructions for the bilberry group included taking dehydrated bilberry (13.3 g/25 ml) three times a day with meals for a cumulative dose of 40 g of bilberry supplement daily. It should be noted that the bilberry contained 2250 mg of anthocyanins. However, participants in the control group were asked not to consume blueberries or bilberries during the study to mitigate confounding variables. Venous blood samples were collected after 10 min of rest following a 6MWT. Lipid panels, inflammatory biomarkers, and heart function tests were obtained at baseline and the end of the eight-week study. Data were analyzed using a two-way ANOVA. The results

showed that hs-CRP levels were not significantly different between groups; however, Ox-LDL levels significantly decreased ($p=0.017$) and the 6MWT increased significantly ($p=0.03$) after eight weeks of bilberry supplement compared to the control group. A limitation of the study was the change in the study design, due to an adverse side effect of the placebo blueberry-flavored powder. The study was terminated, decoded, and redesigned as an open-label and no placebo supplement with a non-dietary intervention arm. This may have introduced participant bias since they may have been aware of the treatment received. In addition, this complication may have affected participant recruitment numbers. Lastly, further studies with female subjects must be investigated using freeze-dried bilberry to reach generalization. These findings demonstrate the benefits of bilberry enriched with flavonoids and its effects on reducing oxidative stress and improving exercise tolerance.

In the next section, a Mediterranean diet assessing flavonoid intake and CVD biomarkers will be reviewed that may offer cardioprotective measures.

EFFECTS OF A MEDITERRANEAN DIET ON CARDIOVASCULAR HEALTH

In a Mediterranean (Med) diet study, researchers studied the effects of polyphenol intake on cardiovascular events (91). A Med diet includes foods rich in fruits and vegetables, grains, and fish which have been associated with improving parameters in cardiovascular health. The study design was a large, parallel group, randomized controlled clinical trial conducted with 4.8 years of follow-up (91). This was a sub-study of a larger study PREDIMED population. The sample size included a total of 1139 (511 men and 626 women) participants ages men 55-80 years and women 60-80 years. Individuals selected for the study had no CVD at enrollment but had three or more CV

related major risk factors such as elevated LDL, obesity, hypertension, type 2 diabetes, etc. There were three study arms including the first arm with a Med diet, dietary changes and advice, and administration of extra-virgin olive oil (EVOO). The second arm included a Med diet, dietary changes and advice, and consumption of mixed nuts (hazelnuts, walnuts, and almonds). The third arm or the control group was provided dietary advice according to the American Heart Association low-fat dietary plan. Each intervention lasted one year. Plasma biomarkers were collected in addition to total urinary polyphenol excretion (TPE). Valid measures such as semi-quantitative food frequency, physical activity, and lifestyle questionnaires were used to measure dietary intake and lifestyle patterns. Data analysis included biomarkers VCAM, ICAM, IL-6, TNF alpha, and MCP-1. Cardiovascular health status included participants' blood pressure, lipid profile (total cholesterol, triglycerides, LDL, and HDL levels), and blood glucose levels. Results after one year indicated that participants with the highest levels of TPE had the greatest decrease in inflammatory biomarkers (ICAM-1, IL-6, and TNF-alpha) in time x group interaction. VCAM ($p < 0.001$), IL-6 ($p = 0.006$), ICAM ($p < 0.001$), MCP-1 ($p = 0.019$), and TNF-alpha ($p = 0.001$) for the Med diet+EVOO and the Med diet+mixed nuts groups all showed significant decreases in these inflammatory markers in comparison to the control group that remained increased from baseline after the 1-year intervention period. Other significant changes after one year of polyphenol intake included a significant decrease in blood pressure within the Med diet+EVOO and the Med diet+mixed nuts groups, but no changes were observed in lipid profiles or blood glucose levels. Overall, there was an increase in polyphenol intake and improved inflammatory and health status markers. In conclusion, these results demonstrate that

diets with higher amounts of polyphenols Med diet+EVOO, and Med diet+mixed nuts and assessed by TPE were associated with lower inflammatory biomarkers. In particular, Med-diet+ EVOO demonstrated a greater effect in reducing inflammatory biomarkers than any other group compared. This study demonstrates that diets enriched with olive oil and nuts have a greater role in CV protection than diets by reducing inflammatory biomarkers that do not contain a high number of polyphenols.

In discussions and applications, many studies presented in this literature review highlighted the association between increased flavonoid intake and changes in the lipid profile (decreased TC, LDL-C, and increased HDL levels), biomarkers of inflammation (CRP, E-selectin, IL-6, ICAM, TNF-A, MCP), oxidative stress (F₂-isoprostanes, ox-LDL), DNA activity repair (OGG1), and improved CV health (blood pressure and increased FMD). For example, Hertog et al.'s (1993) classic study and Medina et al. (2017) both demonstrated that fruits and vegetables enriched with flavonoids in the diet decreased CV events. Using a different approach in a randomized, double-blind, placebo-controlled study, investigators in the Engler et al. (2004) study discovered that high-flavonoid-rich dark chocolate with epicatechin improves endothelial function as evidenced by FMD measurements in a young healthy adult population. Similarly, Khan et al. (2014) found increased FMD when participants (n=66) consumed juice with a high concentration of blackcurrants. Moreover, there was also a significant decrease in F₂-isoprostane levels, which suggests that oxidative stress decreased. However, not every inflammatory biomarker studied decreased consistently across the 10 studies reviewed. For instance, one study with a pomegranate juice intervention did not have any changes in inflammatory biomarkers CRP or E-selectin, but there was a significant decrease in

oxidative stress biomarkers. Regarding the lipid profile results, the findings were consistent with the animal study and all of the human studies. Primarily, triglyceride and total cholesterol levels decreased with high flavonoid intake. Many of the RCTs reviewed in this paper had adequate numbers of participants to achieve power in the studies which were in line with other external clinical trial participation rates. No adverse effects were reported by participants in nine out of 10 studies reviewed. Interestingly, a common theme throughout many of the studies in this review was a lack of generalization and diversity of participants in race/ethnicity and equal representation of male and female participants. Moreover, studies that used FMD and biomarker measures were able to demonstrate a higher level of evidence of improved blood flow and anti-inflammatory properties from flavonoids such as in the chocolate study conducted by Engler et al. (2004). Overall, nutrients enriched with flavonoids supplemented seem to confer cardioprotective, psychological, and improved health benefits.

In summary, there is a paucity of current research investigating the effects of flavonoid intake on CV performance in the military as compared to the civilian population. Consumption of a high flavonoid diet on military performance may benefit soldiers by sustaining or optimizing the warfighter's CV health and readiness. Future research studies should be conducted to identify the effects of flavonoid intakes and their association with CV health and performance. Although human studies are very informative linking berry extracts to health outcomes, cell studies may provide a strong scientific approach to test berry extracts in a controlled, rigorous, and repetitive fashion. This will enhance our understanding of specific mechanisms related to CVD such as oxidative stress. Cell studies are informative in that conditions can be set with minimal

confounding factors to provide reliable mechanism measures of potential health benefits of berry extracts.

SEARCH DESCRIPTION

The following databases were searched on June 2, 2020, for relevant articles in PubMed, CINAHL, and EMBASE. The literature search included combinations of keywords and appropriate subject headings for each database to retrieve articles on flavonoids especially blackcurrant and mood. Results were limited to English. The search strategy identified a total of 661 articles (after the removal of duplicate 415 articles) 246 articles were reviewed.

To ensure each concept was fully represented for blackcurrant, mood, and biomarkers incorporated terms such as “ribes nigrum,” “ribes americanum,” “black currant,” “blackcurrant,” “flavonoids,” “blackcurrant extract,” “grossulariaceae,” “mood,” “depression,” “positive mood,” and “biomarkers.”

LITERATURE REVIEW ON PSYCHOLOGICAL HEALTH (MOOD STATUS)

Another growing area of research is flavonoids and their association with psychological health and mood. Depression rates are increasing in the U.S. and associated underlying factors are CVD, trauma, mental stress, and inflammation (93; 154). Therefore, innovative solutions such as dietary supplementation with flavonoids, and berries, in particular, may offer an alternative approach toward promoting psychological health. This could also positively impact performance and operational readiness.

Mood can be described as a fundamental psychological state that can arise in response to an event (86). The mood is so profound in that it can shape the way we perceive things and impact our behavior, mental, and physical health (8; 86). There are two components to mood: Positive affect (PA) and Negative Affect (NA). PA is the extent to which a person feels enthusiastic, vigor, and alert (151). NA is the extent to which a person feels distressed, angry, nervous, or depressed (151). Depression is one characteristic of mood that affects a large proportion of the population with CVD and is especially prevalent in the military due to the intense performance demands and requirement to always be operationally ready. Therefore, mood and its psychological health impacts are important topics for this literature review. Moreover, potential nutritional strategies for psychological health may have implications to optimize military health.

Depression refers to a state of low mood, lack of vigor, and/or diminished quality of life (15). Depression is the fourth leading cause of disability and impacts greater than 264 million people globally (154). Economically, the U.S. spends approximately \$1 trillion on mental health and treatments (154). If depression is left untreated, depression can lead to suicide. A vulnerable population that is susceptible to depression is the military community. The military community is consistently faced with challenges of high-stress work environments and multiple deployments which have led to higher incidences of post-traumatic stress disorders (PTSD), major depressive disorders (MDD), substance abuse, and other comorbid illnesses (72). In a recent Armed Forces surveillance report, 853,060 active component service members were diagnosed with at least one mental health disorder to include depression (135). Therefore, novel preventive

interventions should be developed to improve mood, since negative mood can be associated with depression.

Overall mental health and well-being can be enhanced through improved mood (8). Currently, there are cognitive-based therapies and pharmacological treatments to address negative moods and depression. Yet, these treatments are limited in their approach mainly due to CNS pharmacological treatments lacking the lipid-solubility of crossing the blood-brain barrier (104). In addition, antidepressant pharmacological treatments such as monoamine oxidase inhibitors (MAO-I), Selective Serotonin Reuptake Inhibitors (SSRIs), and Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs) may have associated side effects including weight gain, insomnia, hypertensive crisis (when taken with foods that contain tyramine) that can lead to increased blood pressure and stroke in some individuals (29; 52). Therefore, novel and safe solutions are needed as a preventive measure and/or adjunctive therapy to reduce depression and improve mood. Dietary supplements with berries that contain flavonoids as a natural fruit-derived bioactive component may provide psychological health benefits for military personnel and positively impact readiness. Currently, emerging research has begun to examine the effects of flavonoids on mood status. At present, there is a research gap in understanding the link between flavonoids and mood, and its underlying cognitive mechanisms of actions. In the limited studies analyzing the relationship between flavonoids and mood, theories have suggested a plausible mechanism of action. Berry flavonoids such as those found in blackcurrants may have the ability to modulate monoamine neurotransmitter activity in the CNS. Monoamine neurotransmitters such as dopamine, serotonin, and

noradrenaline play an instrumental role in regulating mood, learning, memory, appetite, sleep, and other executive functions (157).

Previous studies have also investigated the effects of flavonoids on cognition. The results demonstrated that flavonoids improve memory, attention, and cognitive reaction time (58; 123; 129). However, this literature review will focus primarily on the link between flavonoids and mood. The search revealed only a handful of studies conducted on flavonoids and mood and an even smaller subset of studies investigating the relationship between berry extracts and mood. In particular, berries such as blackcurrants have high antioxidant, oxygen radical scavenging, and vasodilatory properties. This is important for vascular as well as psychological health.

EFFECTS OF MIXED BERRIES AND COGNITION

As evidence mounts on the effects of flavonoid-rich fruits on CV properties, emerging research is examining the impact of berries on cognitive performance. In one study, investigators used a mixed-berry juice concentrate and examined its effects on cognitive function (143). In a randomized cross-over study design, healthy individuals were given the concentrate and the effects on the lipid profile and cognitive function were evaluated. The study sample included 40 men and women, who were instructed to consume a total of 600 ml of a mixed berry or control drink daily with meals over five weeks (102). Inclusion criteria were healthy individuals who did not smoke and who were between the ages of 50-70 years. Exclusion criteria included individuals with a fasting blood glucose > 6.1 mmol/L, food allergies, gastrointestinal conditions, or known cognitive disorders. Cardiometabolic tests, cognitive function ($r = .42$ and $.90$), and anthropometrics were measured. As for cognitive function, the verbal working memory

(WM) test for short-term memory recall and information processing was used. A selective attention (SA) test was used to assess individual spatial perception and attention span on a computer program. Results indicated that after five weeks of berry beverage intake, there was a significant decrease in total cholesterol, low-density lipoprotein cholesterol levels, and insulin levels as compared to the control group. There was also a significant improvement in working memory at the 30-minute test point in the berry group compared to the control group. However, there were no significant differences in inflammatory markers, weight, or blood pressure. Limitations of the study included an unequal number of women and men participants and the lack of blinding the control drink. In conclusion, this was a well-conducted, novel study and the first to investigate both cardiometabolic risk factors and cognitive function after a mixed-berry intervention. It demonstrated the lipid-lowering effect and enhancement of working memory after a mixed berry supplement.

EFFECTS OF BLACKCURRANT EXTRACT ON COGNITIVE FUNCTION

In a different study, scientists used a specific supplement drink, blackcurrant extract versus a mixed berry drink to investigate the effects on cognitive function.

This study was a double-blind, counterbalanced (reduces order effects within a study by reversing conditions), placebo-controlled, and repeated measures design conducted to investigate the effects of two types of blackcurrant extracts (Blackadder juice and DelCyan extract) in comparison to a control drink (150). The effects of blackcurrant supplementation on human cognition, mood, and blood biomarkers were

explored. This intervention was conducted over three days with a washout period of 7 days to ensure that there were no treatment effects between blackcurrant extracts.

The sample population included 36 young healthy adults with BMIs below 35kg/m², ages 18-34 years, and the ability to commit to and complete three practice repetitions of the study test. This was done to minimize practice effects and to ensure that the individuals met minimum standards of cognitive functioning. Exclusion criteria included tobacco use, pregnancy, use of other dietary supplements, and/or use of over the counter or recreational drugs. The treatment drinks included 525 mg of polyphenols per 60 kg from either blackcurrant extract (DelCyan) or 142 ml of cold-pressed blackcurrant fruit juice. The control drink did not contain polyphenols. Dose ranges of polyphenols varied depending on body weight. All treatments were sugar matched and supplemented at the same concentrations for the control drink. Appropriate interventional drinks were prepared for the stability of the polyphenol anthocyanin content and confirmed by analysis with high-performance liquid chromatography. The drinks were frozen at -20 degrees Celsius and aliquoted on the day of use. A third party coded and freshly prepared frozen drinks each morning; the study team was blinded to the coding conditions of the drinks. Participants were required to undergo a total of three study days with a seven-day washout period and a battery of cognitive tests and mood measures on the three study test days. Cognitive tests included the digit vigilance task, Stroop task, and the rapid visual information processing (RVIP) task. The digit vigilance scale measured attention and accuracy of selecting target stimuli. The Stroop test measured cognitive flexibility, inhibition, and attention. The RVIP measured both working memory and attention. As for mood measures, the Bond-Lader and the Visual analog scales were used. The Bond-

Lader analog scale is a routinely used tool in scientific investigations such as nutritional interventional studies. It subjectively measures indirect mood ratings (i.e., alert-drowsy, calm-excited) on a scale of sixteen 100-mm lines. The Visual analog scale subjectively measures how “mentally fatigued” participants are, using anchors such as “not at all” or “extremely” following a task; the more mentally fatigued an individual is, the higher the score. As for the study procedures, once written consent was obtained from participants, they were randomly assigned to a treatment group using a Latin square design to minimize any errors or order effects. Participants fasted 12 hours overnight and refrained from consuming dietary supplements during the study. At each session, venous blood samples were collected at baseline and 150 minutes post-consumption of supplement for measurements of monoamine, prolactin, and anthocyanins. Resting heart rate and blood pressure were measured with an Omron M2 device, and blood glucose levels were also measured. Participants were instructed to consume the drink within five minutes. After a 65-minute rest (the time absorption period of the blackcurrant projected to be detectable in plasma), participants completed post supplement cognitive and mood assessments (70 minutes). Next, participants provided another blood pressure, blood glucose, and blood samples following 150 minutes post-treatment consumption. Changes from baseline differences were calculated for mood, cognitive scores, and biomarkers levels.

The results demonstrated a significant decrease ($p < 0.05$) in mental fatigue following a battery of cognitive tests post supplementation of the Delcyan treatment. This significant effect of the Delcyan group was indicative of positive cognitive performance. The digit vigilance test results of speed were significantly increased at all three study points following the Blackadder juice treatment. No significant post supplement effects

were observed after the DelCyan extract for the digit vigilance test. There was no significant difference among all of the groups for RVIP accuracy. No significant effects were observed for reaction time and false alarms with the Blackadder juice. For the Bond-Lader visual analog, there was a higher alertness rating post supplementation in the DelCyan extract group ($p = 0.02$) in comparison to the control drink group. However, there was no significant effect on calm or content ratings as a result of DelCyan or the Blackadder Juice supplement. There were no significant effects in the results of the Bond Lader tool observed in participants consuming the Blackadder juice.

Blood biomarkers of platelet MAO-B demonstrated a decrease in MAO-B activity ($p < 0.001$) post-Blackadder juice in comparison to the DelCyan treatment where no significant effect was demonstrated. This is a significant finding suggesting that blackcurrant extract (Blackadder juice) may modulate or downregulate MAO-B activity. Plasma anthocyanin concentrations (cyanidin glucoside, delphinidin glucoside, and cyanidin rutinoside) were significantly increased post-treatment in both the DelCyan ($p < 0.001$) and Blackadder juice groups ($p < 0.001$) as compared to the control group. Monoamines blood levels showed an increase in normetadrenaline ($p < 0.001$), a metabolite of neurotransmitters norepinephrine and epinephrine post consumption of the Blackadder juice as compared to both the control and DelCyan treatment. There were no significant effects on monoamine levels as a result of DelCyan treatment. In conclusion, this study indicates that the type of blackcurrant drink may contribute to the observed effects of changes in mood, cognitive performance, and biomarkers. The DelCyan extract was positively associated with increased alertness and less fatigue. This suggests that blackcurrant extract (DelCyan) may be an optimal treatment for acutely increasing

mental alertness and decreasing fatigue. In the Blackadder treatment group, the higher blood glucose levels may indicate that the type of blackcurrant extract has a higher sugar moiety. It could also be due to an effect on glucose metabolism. The Blackadder's effect on increasing speed on the digital vigilance task may indicate that there are acute cognitive benefits associated with its use. Blackadder also demonstrated a key and first finding showing that this concentrate can reduce platelet MAO-B activity. The extract has similar anthocyanin components and properties as the supplement to be used in the proposed blackcurrant study. Blackadder extract may have monoamine oxidase inhibitory properties in the central nervous system that are neuroprotective by increasing available monoamines such as dopamine, serotonin, and norepinephrine. This was demonstrated in the current study with the increase in monoamine blood levels for catecholamines after consumption of the Blackadder juice. Therefore, it is important to further assess the effects of blackcurrant on mood and cognition, as well as exercise performance.

EFFECTS OF NEW ZEALAND BLACKCURRANT JUICE ON MOOD AND EXERCISE PERFORMANCE

In another study, scientists investigated the effects of blackcurrant juice on mood, physiological biomarkers, and treadmill exercise performance (81). The study was a parallel, placebo-controlled, double-blinded design with randomization. The population sample consisted of 40 male and female healthy participants with an age range of 20 to 59 years old. The participants were asked to abstain from foods containing antioxidants and polyphenols from their diet 24 hours before the start of the study. Participants were also asked to refrain from exercise 48 hours before exercise trial day. Health screening

was completed before the initiation of the study. Inclusion criteria were healthy males and females who lived sedentary lifestyles. Exclusion criteria were known fruit allergies, blood-borne diseases, currently taking medications that affect blood properties, inability to walk at a moderate speed confidently on the treadmill, current injury or sustained within the last three months, or chronic heart and breathing problems. The nutritional intervention consisted of blackcurrant juice or a sugar-matched control drink. The treatment drink included New Zealand blackcurrant juice (4.8 mg/kg body weight). The placebo drink had matched glucose, fructose, and vitamin C content relative to body weights respectively. The interventional drinks were prepared 30 minutes before the participant's study day. Both blackcurrant juice and placebo had blackcurrant flavoring added to minimize drink recognition. Before the main trial, individuals were first requested to participate in a pre-trial session. This pre-trial session acclimated participants to the treadmill equipped with an ergometer. Participants performed four-minute walking exercises with up to a 5% incline increase. Heart rate was measured and estimated VO₂ max was calculated during exercise. The subjective visual analog scales (VAS) were also administered after exercise. Both groups had similar height and weight measurements and fitness profiles. Each participant was provided with a standardized meal bar for breakfast and instructed to consume it one hour before starting their respective trial. Measures used for this investigation included the Profile of Mood State (POMS) questionnaire and the VAS. Both scales are reliable and valid tools that have been universally used in mood studies. The POMS scores were calculated by summing the negative (tension, depression, confusion) and positive subscales (vigor and self-esteem) individually. The scores were then subtracted for the total mood disturbance

score. Blood samples were measured for MAO-B activity, blood glucose, lactate, and malondialdehyde (MDA) levels. They were obtained before participants consuming blackcurrant or placebo drink and then the participants were connected to a heart rate monitor. After consuming the beverage, the participants were asked to rest in a waiting area to reflect sedentary activity for one hour. After the wait time, another blood sample was collected, and participants were asked to walk as long as they could at their own walking pace. No other participants were in the same room and time indicators such as clocks were removed. The VAS was administered at the beginning of the exercise test and at three-and five-minute intervals. After the exercise testing, a final blood sample was collected. For analyzing the differences between the interventional groups, two-sample Student t-tests were used for subjective and physiological variables. Repeated ANOVA was used to compare heart rate changes over time and treatment effects. The results demonstrated that blackcurrant juice one hour before exercise significantly decreased MAO-B activity by 90% ($p < 0.001$) and at the end of the two-hour walk. There was no significance reached from pre-exercise to post-exercise levels on heart rate or MDA levels in both the blackcurrant juice and placebo group. The blackcurrant juice group had significantly lower blood lactate levels before exercise ($p < 0.05$) and post-exercise timed consumptions ($p < 0.01$) compared to the placebo group, which did not experience significant differences in X or Y. Overall, the average walking time in the blackcurrant group was 11 minutes longer in comparison to the placebo group, but this difference was non-significant. As for perceived exertion response and mood, both the blackcurrant and placebo group demonstrated similar exertional scores with increases in perceived exertion scores as walk time increased. There was no significant difference in

mood between the blackcurrant and placebo groups. Further studies are needed to further clarify the effects of blackcurrant on mood after exercise.

In conclusion, individuals that consumed blackcurrant juice before a walking exercise decreased MAO-B activity, which may be linked to its effects on exercise and cognitive performance. However, there were no significant differences in mood status between the blackcurrant juice and control groups. Additionally, the blackcurrant group demonstrated a lower dropout rate for exercise performance in comparison to the placebo group which had a higher dropout rate before the two-hour exercise completion time. Although the influence of blackcurrant juice on mood was assessed by MAO-B biomarkers, one limitation of this study was that peripheral measures such as psychomotor vigilance tasks (i.e., reaction time tasks to assess speed or vigilance to stimuli) could have been collected as an additional measure of fatigue. In summary, both this current study and the Watson et al. study demonstrate that dietary supplementation with blackcurrant may modulate cognitive function by inhibiting monoamine oxidase breakdown of neurotransmitters that are associated with mood disorders such as depression. Additional investigations to be reviewed examine the effects of flavonoids on mood, including other fruits that have high amounts of anthocyanins such as wild blueberries (WBB).

EFFECTS OF WILD BLUEBERRIES ON MOOD

Investigators have recently discovered in a randomized, double-blind, placebo-controlled, crossover study that WBB which are high in flavonoids have high antioxidant and anti-inflammatory effects. This study was conducted to determine if the WBB properties improve cerebral blood flow and inhibit monoamine oxidation (66). With this

unique study design, two studies were conducted simultaneously assessing the effects of WBB effects on mood in adults and children. Study one was conducted in an adult population with 21 undergraduate students aged 18–21 years old. Participants were provided with a high polyphenol list consisting of coffee, chocolates, tea, fruits, and vegetables, and asked not to consume it 24 hours before their test session. The participants consumed a WBB drink (250 mg anthocyanins) mixed with 30 g of freeze-dried WBB with 30 ml of low flavonoid concentrate and 220 ml of water during three test sessions. There was a three to seven-day washout period between each test session. The placebo drink was matched to the WBB drink for vitamin C and glucose concentration. Drinks were administered in an opaque cup with black straws to minimize drink recognition. In the second study, 52 school children ages 7 to 10 years old were included. Students were recruited from two local primary schools and written consent was provided by the child's parent or guardian. Food allergies were also confirmed by the child's parent or guardian, and there were no fasting requirements placed on this population. The intervention drinks were identical in the second study to the drink contents in the adult study with the exception that 170 ml of water was added to increase drink palatability. The children underwent a battery of cognitive tests assessing verbal learning skills, reading efficiency, etc. to ensure that there were no learning or attentional deficits. Inclusion criteria were healthy children and young adults. Exclusion criteria included food allergies and medical conditions such as heart disease, diabetes, liver disease, etc. The adult population was asked to refrain from consuming flavonoids provided on a common drink and food list given to participants before testing days. There was no indication that food records were obtained. There was a total of three study test days.

The first session was a practice session to minimize practice effects. All three test sessions took place in the morning with a minimum of a three-day washout period between test sessions. Participants also received the placebo drink at their first session, and they were administered either of the Positive and Negative Affect Scales (PANAS-Now or PANAS-C) as a baseline measure of mood. After the drink was consumed, participants were instructed not to exercise, drink, or eat. Two hours after the drink consumption, participants returned to the laboratory for additional mood measures. Measures used to assess participant mood in both of these studies included the Positive and Negative Affect Schedule (PANAS). The PANAS is a valid and reliable instrument composed of 20-items (10 positive affect and negative affect) to indicate depression symptoms. For example, high positive affect suggests a positive mood, and low positive affect indicates depression symptoms. Scores were calculated using a Likert scale. Overall positive and negative mood responses were individually scored to indicate the degree of positive or negative mood states of each participant. It should be noted that in the adult study, the PANAS-NOW scale was used. The PANAS-C was used for the child study. The PANAS-C is a validated scale that includes the same 20 questions as the PANAS-NOW, but with additional 10 child-friendly synonyms capturing mood measures specific to this population. The PANAS-C scale was used in this population following the same procedural steps as the adult population. In the first study, those taking the WBB drink had significantly increased positive affect scores, which suggests enhanced mood ($p = 0.001$) from baseline. There was a slight decrease in negative affect scores from pre-intervention to post-intervention following the WBB consumption, however, there was no significant difference in either the placebo or the WBB group. In the second study, there

was a significant increase in positive affect scores for mood after consumption of the WBB drink ($p = 0.019$). There was no significant effect after consumption of WBB on negative affect for either the placebo or WBB group. In conclusion, these findings suggest WBB can have a positive effect on mood across various ages. This study was the first to demonstrate the effects of WBB on mood and may provide initial insight for improving depressive disorders. There were some limitations of the study. Plasma biomarkers were not measured so it is difficult to link the underlying changes in physiology that may be associated with the mood changes. In addition, food records were not obtained which would validate participant dietary compliance.

In summary, the randomized clinical controlled trials reviewed in this literature review focused on the effects of flavonoids on psychological health and cognitive function. The results collectively showed that various fruit sources of flavonoids such as blackcurrant and wild blueberries can improve mood and decrease fatigue. Watson et al. (2015) and Lomiwes et al. (2019) similarly demonstrated that the biomarker MAO-B indicative of dopamine metabolism was reduced in those individuals consuming flavonoid-rich Blackadder and blackcurrant juice concentrates. Alertness is also enhanced with the DelCyan preparation, where Blackadder improves performance on the digit vigilance task. Therefore, the specific types of blackcurrant beverages may be affecting different pathways in the brain. Interestingly, both the Lomiwes et al. (2019) and the Khalid et al. (2017) studies demonstrated improved mood status using different validated mood measures of POM and PANAS in the child and adult populations. However, none of these studies measured inflammatory or oxidative stress biomarkers which would have provided important information about physiological mechanisms.

These key biomarkers are imperative to our understanding of the role of fruit flavonoids on CV function and psychological health.

Collectively, all of these studies in three model systems (cell, human, and animal) have demonstrated that berry flavonoids have promising health benefits, even minimizing the potential of developing atherosclerosis. Specifically, the strengths of these above studies are that CV, cognitive, and psychological biomarkers were able to be assessed in a human model. Overall results demonstrated that mixed berries, blueberries, and different blackcurrant concentrates were associated with various effects of reduced cholesterol, LDL, insulin, and improved cognitive performance and mood measures. However, a limitation with these studies is the consistency of berry extracts on end outcomes in comparing results across these various studies. For example, not all studies used similar populations, measures, berry extract concentrates and doses, or biomarkers. Therefore, more clinical trials are needed to generate a solid base for consistent results of berry extracts. Therefore, this study addresses the effects of berry flavonoids on cardiac and neural cell line models, building on our current scientific knowledge and potentially informing future clinical trials that will develop a novel strategy to improve CV and brain health in the warfighter. Additionally, consistency of conditions and end outcomes can be performed repeatedly using berry extracts on cell models. As an outcome, the proposed investigation will determine the effects of a dietary supplement, blackcurrant, on oxidative stress as a mechanism for atherogenesis.

CHAPTER 3: Methods

INTRODUCTION

In this chapter, the study methods were detailed including how the study was conducted, what cell models were be studied, where the study took place, what study design was used, what instruments and measures were used, and what procedures were carried out to address the research questions presented in chapter one. An understanding of the proposed methods in this chapter was provided for clarity on how the goals of this current research study were achieved and to amplify the differences from earlier studies presented in chapter two. This study assessed the effect of a dietary supplement with blackcurrant extract on oxidative stress in cardiac and microglia cells. As a secondary outcome, independent active compounds delphinidin-3-rutinoside and cyanidin 3-glucoside were assessed to determine if these individual components were factors in berry flavonoid performance by measuring oxidative stress.

MATERIALS AND METHODS

Cell Culture

The effects of berry extracts and their major component compounds on cell survival and ROS were examined in two different cell types—HL-1 cardiomyocytes and BV-2 microglial cells.

HL-1 Cardiomyocytes

To further understand the role of berry flavonoids in the CV system, HL-1 cardiomyocytes were selected as the cell line of interest. Various cardiomyocyte cell

lines have been previously been used to test cardiac biology (152) which has relevance for the development of atherosclerosis and CVD. HL-1 cardiomyocytes were first derived from atrial myocardial cells expressing the SV40 T antigen by Claycomb (25). These cells express many of the features of differentiated cardiomyocytes and express many cardiomyocyte-specific genes through an indefinite number of passages ((25). HL-1 cells were maintained in supplemented Claycomb medium at 37° C in a humidified incubator with 5% CO₂. Supplemented Claycomb medium (Sigma) contains fetal bovine serum (10% v/v), penicillin/streptomycin (1000U/ml), norepinephrine (0.1 mM), and l-Glutamine (2mM). Cells were grown in T75 flasks coated with gelatin and fibronectin until 90% confluency and then passaged into 96 well plates for treatments and experiments. Cells were typically sub-cultured twice a week using 0.05% Trypsin/EDTA for dissociation.

BV-2 Microglial cells

To further understand the role of berry flavonoids on brain health, BV-2 microglia were selected as the cell line of interest. These cells have previously been used to test oxidative stress (83; 130; 131) which has relevance for the development of neuroinflammatory diseases and cognitive decline associated with oxidative stress and status of brain health. BV-2 cell models were generated by infecting a mouse microglial cell culture with an oncogene J2 virus which can exhibit inflammatory properties (14). BV-2 cells were maintained at 37° C in a humidified incubator under 5% CO₂ in BV2 media consisting of DMEM supplemented with fetal bovine serum (10% v/v) and penicillin/streptomycin (10 ml), and DMEM. Cells were grown in T75 and T150 flasks

until 90% confluency and then split into 96 well plates before treatments and experiments. Cells were subcultured once a week using 0.25% Trypsin/EDTA, BV2 microglia media, and Phosphate Buffered Saline (PBS) for cell wash. Cells were plated on 6 well or 96 well plates for experiments.

BERRY EXTRACTS

Concentrated berry extracts from the following berries: blackcurrant, aronia berry, cranberry, elderberry, cherry, and berry blend (composition of mixed berries) were obtained. (Artemis International, Inc., Ft Wayne, IN) Different berry extracts have different flavonoids. The major flavonoid in each berry is listed in Table 1. A Certificate of Analysis was provided for berry extracts to ensure the product's content and purity. Dried berry extracts were stored at room temperature and protected from light. Berry extracts were dissolved in water (100 mg/mL) and stored in the freezer at -20° C until experimentation. Berry extracts were added to the culture media for the cell being tested at specific concentrations as noted for each experiment (Appendix A).

TABLE 1 Major Flavonoids in Berry Extracts

Berry Extracts	Major Flavonoid
Blackcurrant	Delphinidin-3-Rutinoside
Aronia berry	Cyanidin 3-Glucoside
Cranberry	Cyanidin 3-Glucoside
Elderberry	Cyanidin 3-Glucoside
Cherry	Cyanidin 3-Glucoside
Berry blend	Cyanidin 3-Glucoside

In a subset of experiments, we compared the effectiveness of major components of the berry extracts—including delphinidin-3-rutinoside (Sigma, cat# PHL80735, MO, USA) and cyanidin 3-glucoside (Sigma, cat# PHL89616, MO, USA). These compounds were used in parallel experiments with berry extracts to assess the relative antioxidant effects that might be attributable to each (Appendix B).

MEASUREMENTS

Cell Viability Assay

To determine the toxicity of the various berry extracts, the Cell Health Assay Kit (CellSignal Technology; Cat # 13837, MA, USA) was used. Briefly, cells were plated in a 96 well plate and incubated with berry extracts at different concentrations ranging from 0.1- 100 $\mu\text{g/mL}$ in cell culture media for 1 hour. Calcein-AM fluorescent

dye was added to the cells at a concentration of 1 μ M to label living cells for 30 minutes. The membrane-permeant calcein-AM dye was rapidly de-esterified in living cells with active esterase, where it becomes trapped. The magnitude of cellular fluorescence is proportional to the number of living cells. The Cell Health Assay Kit had good specificity and provided a sensitivity marker in that it was able to detect at a range from 500-50,000 cells per well. Calcein fluorescence elicited a wavelength of 485 nm and was measured at 520 nm by using a microplate fluorescence reader (Appendix C).

Oxidative Stress Assay

To assess cellular oxidative stress, the CellROXTM Green Assay (Invitrogen, cat. # C10444, OR, USA) was employed following the manufacturer's protocols. CellROXTM green is a weakly fluorescent dye in its native state, but upon oxidation, its fluorescence increases markedly, and it binds to DNA. The magnitude of cellular fluorescence was proportional to the degree of oxidative stress in the cell. Briefly, cells were plated in a 96 well plate and incubated with menadione (vitamin K₃; Cayman Chemical, cat # 15950) to induce oxidative stress in the presence or absence of berry extracts at different concentrations as noted in the text. CellROXTM dye was added to the cells according to the manufacturer's protocols for 30 minutes. Following washing with PBS, resultant fluorescence elicited a wavelength of 485 nm and was measured at 520 nm using a microplate fluorescence reader. In all experiments, a canonical antioxidant (1 mM N-acetyl-cysteine) was used as a positive control to ensure the quality of the results (Appendix D).

Western Blot

Western blot is a technique that was used to identify specific proteins by their molecular profile through the separation of their molecular weight and size (79). Bands were generated through a membrane transfer from gel electrophoresis to represent the markings of a specific protein under investigation. For the procedure, cells were washed with 2mL of ice-cold PBS and aspirated; and then 300 ug/ml of lysis buffer with protease inhibitor was added to the cells and they were placed in a rocker at 4°C to separate the proteins. Next, cells were centrifuged for eight minutes at 8,000 RPM. The resultant supernatant lysate was used for the western blot analysis and the pellet was discarded. INOS primary antibody was used as a 1:1000 dilution (BD Biosciences, USA/ lot number 7334828). The western blot bands were analyzed on the c280 azure biosystems reader.

Nitrite Quantification

To assess the amount of nitrite, present in the cell culture medium, a nitrite quantification assay was used. NO is an important signaling molecule that is naturally produced in the human body; it is unstable and has unpaired electrons that produce free radicals (48). NO also has a significant role in vascular health by producing vasodilation. NO is also an important molecule of interest in neuronal cells due to its involvement in oxidative stress processes and inflammation. NO is involved in several pathological and physiological immune responses which include inflammation, tissue repair, neurotransmission, and endothelial vasorelaxation (48). To determine the

quantification of NO as a direct measure, the Griess Reagent System was employed. This system was based upon a chemical reaction that can detect NO in the *in vitro* setting. NO was of particular interest because berry extracts have been previously shown to modulate NO production (130). Briefly, cells were plated in a 96 well plate and incubated overnight for 24 hours as pretreatment with their respective berry extracts or bioactive compounds in various dose concentrations. The primary berry extracts that were measured included blackcurrant and cranberry extracts. These whole berry extracts were compared to C3G or D3R in various dose concentrations (ranging from 1- 100 µg/mL in cell culture media). A nitrite standard reference curve was produced and compared to the experimental samples. Absorbance was measured between 520nm and 550nm using a microplate reader for Griess Reaction.

Data Analysis

Statistical analysis was performed using Microsoft Excel (version 10 for windows) and Graph Pad (version nine software). Data were analyzed for both the primary and secondary aims using ANOVA and t-tests comparing the differences between the means of the berry and control groups. The primary outcome was induced oxidative stress. Tukey's HSD or Dunnett's tests were performed as appropriate if overall significance was observed. The results were considered statistically significant if the p level was <0.05. There were no privacy issues with data considering cells have no sensitive or identifying data. Data were primarily stored and managed in Microsoft Excel (version 10). To ensure experiment validity, a control was put in place such as a known potent antioxidant to compare with the berry extracts. Additionally, experiments were

repeated no less than three times to ensure data integrity. Data were expressed as mean with the standard deviation.

ETHICAL CONSIDERATIONS

There were no ethical concerns or storage of data concerns within this study.

QUALITY ASSURANCE

Cell models were maintained at appropriate levels and protocols were followed stringently. Active compounds, reagents, and berry extracts were stored at appropriate temperatures and settings. Sterile techniques were adhered to avoid cell culture contamination and to optimize results. Independent double verification of active compounds was conducted as appropriate to ensure treatment precision. Data analysis was conducted with statistical consultation to assist in reducing bias in the analysis.

CHAPTER 4: Results

Oxidative stress and its pathological downstream effects are likely involved in a wide range of health conditions, including CV and neurological diseases. Berry flavonoids have been found to reduce inflammation as well as oxidative stress processes within the CV and central nervous systems (45; 58; 99; 146). Although some researchers have explored the effects and composition of certain types of flavonoids (e.g., those found in vegetables, chocolate, legumes, and spices such as curcumins), few studies have addressed the role of berry flavonoids' in reducing oxidative stress on cardiomyocyte and microglial cells. Therefore, we examined the effects of berry flavonoids on specific cell models (HL-1 cardiomyocytes and BV-2 microglial cells) to determine their potential health benefits of decreasing oxidative stress in the present study.

EFFECTS OF BERRY EXTRACTS ON OXIDATIVE STRESS WITHIN HL-1 CELLS

To start, HL-1 cardiomyocytes were pretreated with six berry extracts (blackcurrant, aronia berry, cranberry, elderberry, cherry, and a berry blend extract mixture) to determine if they inhibited the generation of ROS. Cells were pretreated for 1 hour with each of the berry extracts (1 mcg/mL). In the first experiment, the berry extracts were tested to determine if they had any effect on basal oxidative stress in HL-1 cells. As a control, cells were also treated with a potent antioxidant, N-acetyl cysteine (NAC) at a concentration of 1 mM. Oxidative stress was assessed using CellROX™ as described in the Methods. As shown in Figure 3a, neither NAC nor any of the berry

extracts affected CellROX™ fluorescence consistent with the conclusion that there is little or no oxidative stress in HL-1 cardiomyocytes in the absence of a stressor.

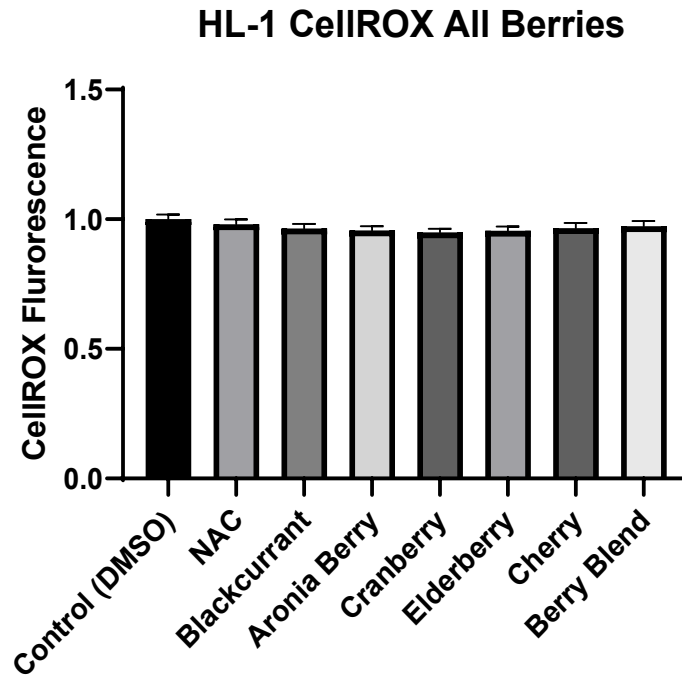


Figure 3a. Effects of Berry Extracts on Oxidative Stress in HL-1 Cells

Shown are results of CellROX™ fluorescence from experiments (n = 3) demonstrating the effect of berry extracts on basal oxidative stress in HL-1 cardiomyocytes. Neither the potent antioxidant, NAC, nor any of the berry extracts had a significant effect on CellROX™ fluorescence in the absence of an oxidative stress-inducing agent.

Next, the ability of the berry extracts to counteract the oxidative stress induced by menadione (also known as vitamin K3) was determined. As shown in Figure 3b, K3 induced a marked increase in CellROXTM fluorescence. As expected, pretreatment with the potent antioxidant, NAC, significantly inhibited ($p<0.001$) K3-induced oxidative stress. Among the berry extracts, only blackcurrant extract significantly reduced ($p=0.037$) oxidative stress induced by K3. In contrast, none of the other berry extracts inhibited K3-induced oxidative stress. For example, oxidative stress in the presence of K3 + cranberry extract was nearly identical to treatment with K3 alone. Based on these results, blackcurrant and cranberry extracts were selected for further investigation to better understand the differences between the radical scavenging activity of these particular berry extracts and their components.

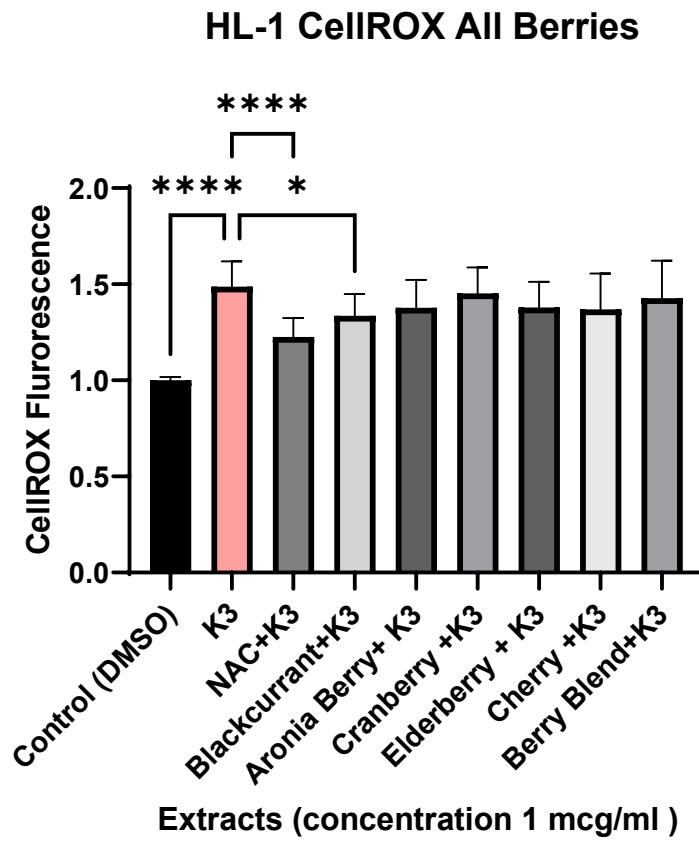


Figure 3b. Effects of Berry Extracts on K3-Induced Oxidative Stress in HL-1 Cells

****Significant differences at $p < 0.0001$, * $p < 0.05$.

Shown are results of CellROXTM fluorescence from experiments ($n = 3$) demonstrating the effect of berry extracts on menadione (Vitamin K3) induced oxidative stress in HL-1 cells. In each experiment, CellROXTM fluorescence was normalized to the Control (DMSO vehicle). K3 (100 μ M) elicited a significant increase ($p < 0.0001$) in CellROXTM fluorescence indicating a marked increase in oxidative stress. The potent antioxidant N-acetylcysteine (NAC, 1 mM), significantly reduced ($p < 0.0001$) K3-induced oxidative stress, whereas blackcurrant extract alone among all of the berry extracts markedly inhibited oxidative stress ($p < 0.05$).

EFFECTS OF BLACKCURRANT AND CRANBERRY EXTRACTS ON HL-1 CELL VIABILITY

Increases in oxidative stress are associated with increased cell death. Therefore, cell viability with HL-1 cardiomyocytes was determined. The results showed that K3 induced a significant increase in cell death as assessed by calcein-AM staining as described in the methods. As shown in Figure 4, treatment with K3 induced significant ($p < 0.0001$) cell death, whereas NAC inhibited ($p = 0.0006$) K3-induced cell death. Interestingly, neither blackcurrant nor cranberry extracts (100 mcg/mL) exhibited significant effects on cell viability. However, it was apparent that blackcurrant extract caused a modest improvement in viability, whereas cranberry extract appeared to have no effect at all, in agreement with the results observed for oxidative stress.

In addition, there was an observed correlation with NAC and blackcurrant (100 mcg/mL) in the presence of menadione-induced oxidative stress which indicated preservation of greater cell viability than cranberry extract (100 mcg/mL) in the HL-1 cells. NAC is a potent antioxidant, and blackcurrant extract demonstrates similar properties to NAC under menadione-induced oxidative stress. Similar cell viability tests were conducted using another cell model with BV-2 cells (microglia cells) under the same conditions of menadione-induced oxidative stress using K3 as was used in the HL-1 cells (Figure 4). Cranberry extract (100 mcg/mL) significantly increased cell viability in comparison to the K3 only group and blackcurrant (100 mcg/mL) + K3 group (Figure 4).

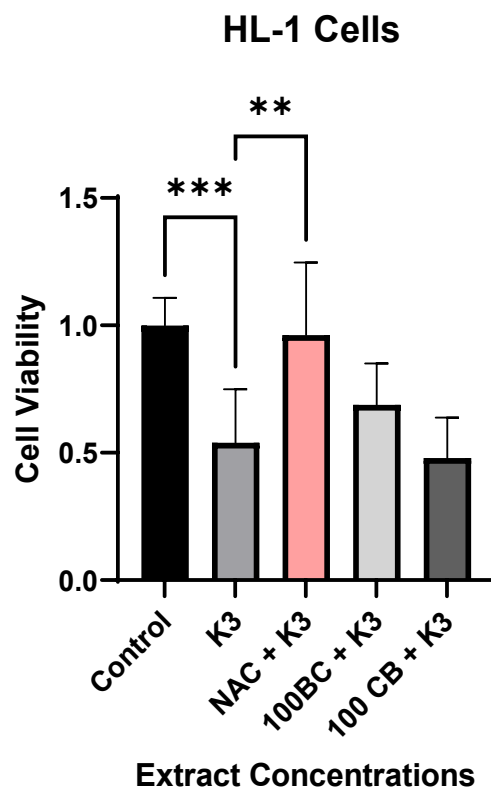


Figure 4. Effects of Berry Extracts on Cell Viability in HL-1 Cells

***Significant difference at $p < 0.001$, ** $p < 0.01$.

Shown are normalized results of calcein-AM staining of live cells following treatments as indicated. As expected, K3 induced a significant reduction ($p < 0.001$) in cell viability, whereas 1 mM NAC significantly inhibited K3-induced cell death ($p < 0.01$). Although neither berry extract significantly affected cell viability, blackcurrant extract (100 mcg/mL) offered modest protection when compared to cranberry extract which offered no protection from cell death caused by K3.

EFFECTS OF BLACKCURRANT AND CRANBERRY EXTRACTS ON OXIDATIVE STRESS IN BV-2 CELLS

To determine whether the effects of blackcurrant and cranberry extracts were generalizable to other cell types, the effects of the berry extracts were assessed in parallel experiments in BV-2 microglial cells. Microglial cells are the resident immune cells in the central nervous system, and dysregulation of these cells can affect brain health. Because of the distinctly different results with blackcurrant and cranberry extracts on HL-1 cells, these extracts were the focus of the following experiments. At concentrations of 10 mcg/mL and 100 mcg/mL, blackcurrant extract suppressed K3-induced oxidative stress. Interestingly, at a concentration of 1 mcg/mL, there was no effect observed with blackcurrant extract. Conversely, at all concentrations tested (1, 10, and 100 mcg/mL) cranberry extract reduced oxidative stress. Neither extract affected basal oxidative stress in the cells (*data not shown*). When comparing these results with those obtained in HL-1 cells where only the 1 mcg/mL dose was tested, it is apparent that the effects of blackcurrant and cranberry extracts in the two cell types are markedly different. Blackcurrant is the most powerful antioxidant in HL-1 cells, whereas cranberry extract is more potent in BV-2 cells.

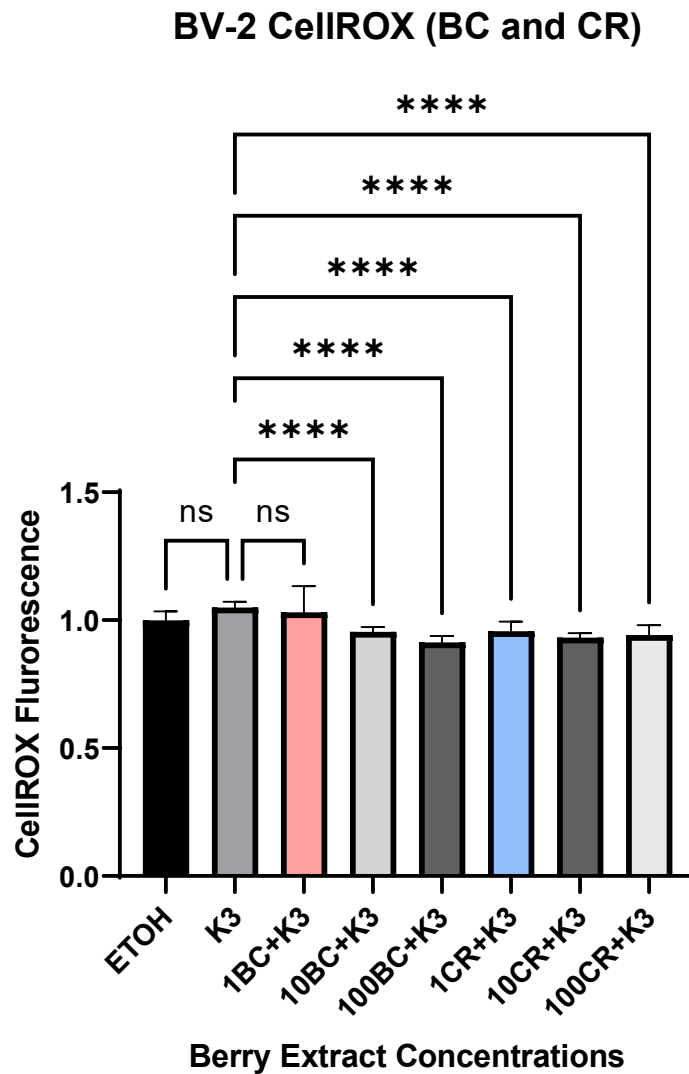


Figure 5. Effects of Berry Extracts on Oxidative Stress in BV-2 Cells

****Significant differences at $p < 0.0001$, ns indicates not significant.

This figure depicts the results of CellROXTM fluorescence from experiments ($n = 12$) showing the effect of berry extracts on menadione (Vitamin K3) induced oxidative stress in BV-2 cells. In each experiment, CellROXTM fluorescence was normalized to the control (ETOH vehicle). K3 (100 μ M) in comparison to ETOH was non-significant but elicited a marked increase in CellROXTM fluorescence indicating an increase in oxidative stress. Except for the lowest concentration of blackcurrant (BC) extract (1 mcg/mL), all other berry extracts significantly suppressed oxidative stress. The difference in effects between the blackcurrant and cranberry (CR) concentrations at the 1mcg/ml dose are highlighted in pink and blue. 1 CR+K3 shows a significant decrease in oxidative stress, whereas the 1BC+K3 did not affect the BV-2 cells.

EFFECTS OF BLACKCURRANT AND CRANBERRY EXTRACTS ON OXIDATIVE STRESS IN BV-2 CELLS

The effects on cell viability following oxidative stress were also tested using the calcein-AM staining assay. The results of this experiment are shown in Figure 6.

Cranberry extract (100 mcg/mL) was the most effective berry extract in preserving cell viability in the presence of K3, suggesting that the antioxidant properties are coupled to improved cell survival. Interestingly, however, neither blackcurrant extract (100 mcg/mL) nor the potent antioxidant, NAC, provided a protective benefit, even though they inhibit oxidative stress.

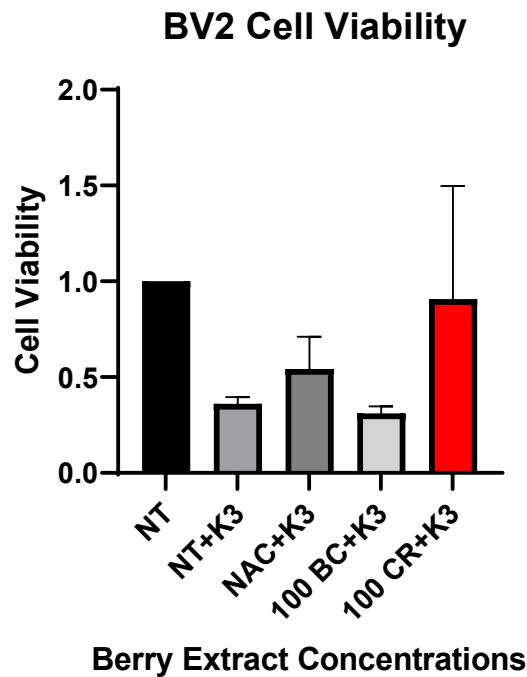


Figure 6. Effects of Berry Extracts on Cell Viability in BV-2 Cells

This figure demonstrates menadione (K3)-induced oxidative stress which decreased cell viability in comparison to the non-treatment group. As for treatment groups, NAC+K3 and Blackcurrant (100 mcg/mL) + K3 were non-significant for cell viability to K3 only. Although cranberry extract+K3 (100 mcg/mL) did not show significance in cell viability compared to the other treatment groups, it had the highest average cell viability within the BV-2 cells. Due to the large standard deviation in one group, group means were compared by Welch's ANOVA followed by Dunnett's pairwise comparisons.

COMPARISON OF DELPHINIDIN-3 RUTINOSIDE AND CYANIDIN-3-GLUCOSIDE ON OXIDATIVE STRESS IN BV-2 CELLS

To examine the possible basis for the disparate results of the different berry extracts, the different molecular makeup of the extracts was considered. Based on the certificate of analysis, it is apparent that the major anthocyanin found in blackcurrant is delphinidin-3-rutinoside (D3R), whereas cyanidin-3-glucoside (C3G) is the principal component in cranberry extract. It was hypothesized that the different behavior of the extracts might be related to the different anthocyanins. Therefore, the effects of the individual components on oxidative stress induced by K3 were assessed. The results of this experiment are shown in Figure 7. As expected, K3 induced a significant increase in oxidative stress as measured by CellROX fluorescence and this was inhibited by both blackcurrant and cranberry extracts at 10 and 100 mcg/mL concentrations. Interestingly, the most effective antioxidant among the components was cyanidin-3-glucoside (C3G). Delphinidin-3-rutinoside (D3R) was less effective as an antioxidant.

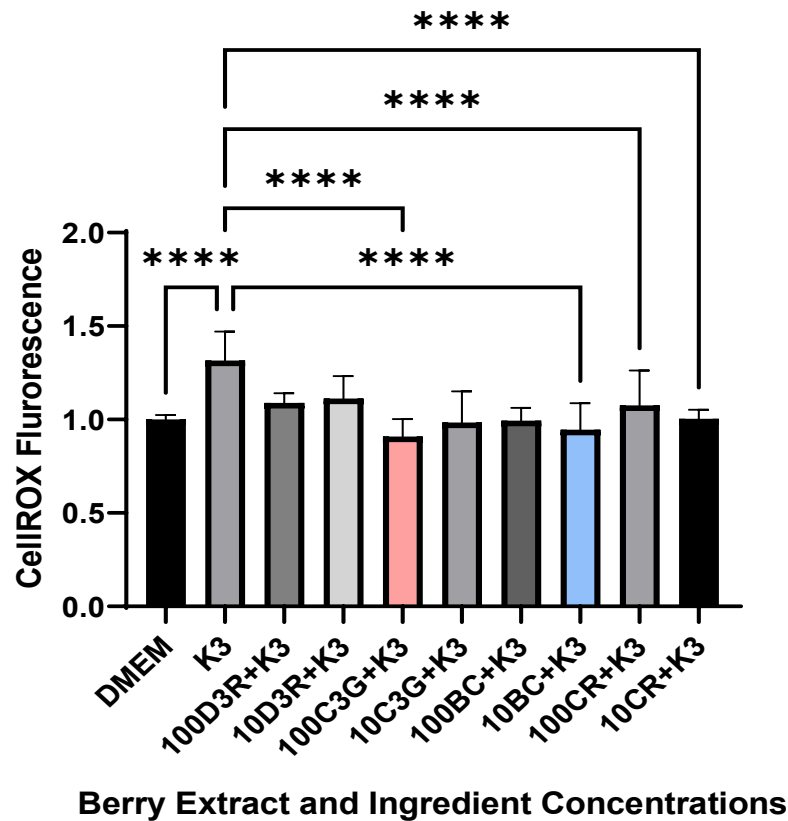


Figure 7. Effects of Berry Extracts and Active Compounds on Oxidative Stress in BV-2 Cells

****Significant differences at $p < 0.0001$.

Shown are results of CellROXTM fluorescence from experiments ($n = 3$) demonstrating the effect of berry extracts on K3-induced oxidative stress in BV2 cells. The diagram above shows a significant induction of K3-induced oxidative stress in BV-2 microglia cells ($p < 0.0001$) compared to the control group (DMEM). In comparison to K3, all the combination extracts + K3 were significantly different from the K3 control group ($p < 0.0001$). However, the combination + K3 groups had the greatest mean difference which demonstrates that the most effective at reducing oxidative stress in microglia cells were the 100C3G+K3 ($p < 0.0001$) and the 100BC+K3 groups ($p < 0.0001$).

EFFECTS OF BERRY EXTRACTS ON iNOS EXPRESSION IN BV-2 CELLS

Increases in inducible nitric oxide synthase (iNOS) are associated with the activation of microglial cells and initiation of the immune response. Lipopolysaccharide (LPS) is a common method employed to initiate the immune response of BV-2 cells and has been shown to induce an increase in iNOS expression (137). To determine whether blackcurrant or cranberry extracts interfere with this process, the ability of LPS to activate BV2 cells and induce iNOS expression was compared in the presence or absence of berry extracts. Representative western blot data are shown in Figure 8. As expected, LPS (100 ng/mL for 16 hrs) induced robust expression of iNOS and the berry extracts did not affect iNOS expression in the absence of LPS. Interestingly, although blackcurrant did not induce iNOS expression on its own, it potentiated the response to LPS in a concentration dependent fashion. Conversely, cranberry extract inhibited LPS-dependent iNOS induction at lower concentrations, but not at the highest concentration tested. These results indicate that the different berry extracts exert markedly different effects on BV-2 cells with unique results on iNOS induction (Figure 8).

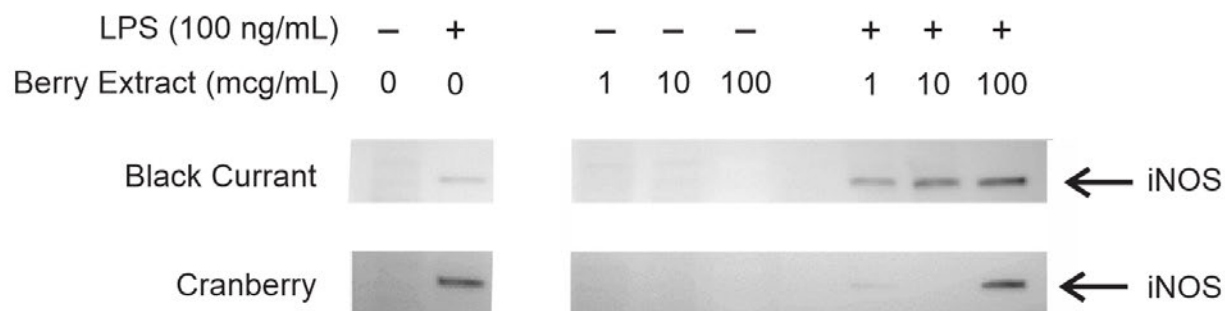


Figure 8. Effects of Berry Extracts on iNOS Expression in BV-2 Microglia Cells

Shown are Western blots from BV2 cells treated with LPS (100ng/mL) $\pm$ berry extracts for 16 hours. LPS induced modest induction of iNOS expression. Berry extracts did not affect iNOS expression in the absence of LPS; however, LPS-induced iNOS induction was potentiated in the presence of blackcurrant in a concentration dependent manner. In contrast, cranberry extract inhibited the induction of iNOS expression at lower concentrations, but not at higher concentrations.

EFFECTS OF BERRY EXTRACTS ON LPS-INDUCED NITRIC OXIDE IN BV-2 CELLS

The induction of iNOS leads to the production of nitric oxide (3; 77). Since blackcurrant extract appears to sensitize the cells and increase the expression of iNOS in the presence of LPS, it would be expected that nitric oxide synthesis and release would be increased as well. Conversely, cranberry extract would be expected to inhibit nitric oxide production. To test this, nitric oxide released into the media by BV-2 cells was measured using the Greiss assay as described in the Methods section. The results of this approach were variable. As expected, LPS induced a marked increase in NO production when compared to control, untreated cells ($p=0.0027$). There were no statistically significant effects of the berry extracts at any concentration. Consistent with the effects on iNOS expression, the blackcurrant extract (100 mcg/mL) blackcurrant+ LPS did induce the largest increase in NO production relative to the no treatment group. The cranberry extract, however, did not inhibit NO production at any concentration, even though it markedly impaired iNOS induction.

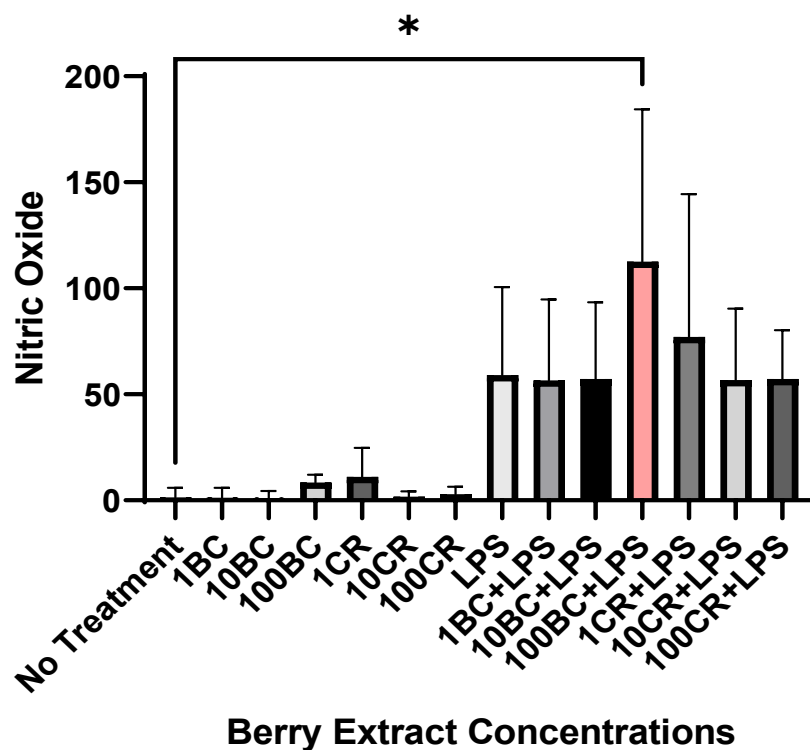


Figure 9. Effects of Berry Extracts on NO in LPS-induced BV-2 Cells

*Significant difference at $p<0.05$.

The graph depicts the nitric oxide (NO) concentrations secreted from BV-2 microglial cells in response to LPS in the presence or absence of blackcurrant (BC) or cranberry (CR) extracts. Consistent with the previous western blot data, blackcurrant extract (100 ug/ml) blackcurrant+ LPS induced the largest increase in NO production relative to no treatment ($p<0.05$). Surprisingly, cranberry extract did not affect LPS-induced NO production.

CHAPTER 5: Discussion

Oxidative stress is a key mechanism linked to endothelial dysfunction, decreased NO, and promotion of inflammatory cytokines such as tumor necrosis factor-alpha and interleukin factors, all of which can lead to pathophysiological processes of atherosclerosis in heart disease and neurological conditions (65; 146). Oxidative stress and its downstream pathology have been a long-standing issue not only within the civilian force but also within the Warfighter community and its mission on readiness. Statistics have shown that increases in heart disease and mental health conditions are steadily rising within the military both in garrison and theater (24; 35; 105; 135).

Flavonoids such as those found in berries could potentially benefit this population's health through their reported properties of inhibition of oxidative stress, regulation of NO, and cardio- and neuroprotective mechanisms (2; 107). Berry flavonoids have been previously shown to reduce inflammation as well as oxidative stress processes in both the CV and central nervous systems (45; 58; 99; 146). Therefore, berry flavonoids were investigated in a systematic approach in specific cardiomyocyte and microglial cell models (HL-1 and BV-2 cells) to determine their potential effects and roles in oxidative stress.

Previous investigations have demonstrated that flavonoids exert antioxidant effects that confer a cardioprotective effect against oxidative stress (20; 53; 96). These studies have pointed to the phytochemicals present in many of whole foods and the benefits that can be derived from a variety of food sources (53). For instance, individual foods rich in flavonoids such as blueberries, wine, apples, and chocolate are associated with decreased incidences of CVD (96). Moreover, chocolate which contains epicatechin

has vasculoprotective effects such as suppression of platelet activity, activation of endothelial nitric oxide synthase, and increased FMD in a healthy population (36; 37). These foundational studies led to closer observations of specific flavonoid properties and interest in their antioxidant properties on oxidative stress, inflammation, and the need to target various chronic diseases with nutritional interventions in support of CV and brain health.

Studies undertaken in cells can help us understand the underlying mechanisms of berry flavonoids. Antioxidant properties of berry extracts *in vitro* include investigations which are relevant to human health such as inhibiting oxidative stress, regulating iNOS and NO production , and determining the effects of berry bioactive compounds in a dose response manner (57; 82; 126; 130; 131). The experiments in this study add to this evidence base and point out some critical observations that should be considered in research on the CV and brain health benefits of berry flavonoids.

COMPARING THE EFFECTS OF BERRY EXTRACTS ON OXIDATIVE STRESS AND CELL VIABILITY IN HL-1 AND BV-2 CELLS

In this study, the effects of berry extracts on oxidative stress and cell viability were measured in both HL-1 cardiomyocytes and BV-2 microglia, with distinctly different outcomes. Although blackcurrant extract was the most effective of the extracts at reducing K3-induced oxidative stress in HL-1 cells, it was less effective at reducing oxidative stress in BV-2 cells. Similarly, blackcurrant extract was more effective than cranberry extract at preserving cell viability in the face of oxidative stress in HL-1 cells, whereas the opposite was true in BV-2 cells. This suggests that not all cell types respond to the extracts in the same way and that all extracts may not share the same mechanism of

action. This may be attributed to the antimicrobial properties of cranberry through its bioactive compounds such as quercetin and its higher concentrations of proanthocyanidins.

N-acetyl-cysteine (NAC), a potent antioxidant, was used as a control in cell viability assays. NAC, a precursor of glutathione and derivative of the amino acid cysteine, serves to regulate intracellular redox signaling of cell survival and death (159). In a previous study, NAC was used to pre-treat cells as an antioxidant under H₂O₂ induced oxidative stress conditions. Oxidative stress was significantly reduced and glutathione levels were significantly increased in H9C2 cardiomyocyte cells (78). This is consistent with the findings in the present study where NAC was most effective in preserving cell viability in HL-1 cells. The observation that blackcurrant was the most effective inhibitor of oxidative stress and preserving HL-1 cell viability suggests that both NAC and blackcurrant may have similar cellular antioxidant pathways of promoting cell survival through downregulation of cell apoptosis. It also suggests that blackcurrant extract has free radical scavenging activities and is capable of attenuating oxidative stress in cardiomyocyte cells.

In parallel experiments using BV-2 cells, markedly different results were observed with blackcurrant and cranberry extracts. Interestingly, neither NAC nor blackcurrant extract were effective at preserving cell viability in BV-2 microglia. Although the results did not reach statistical significance for cranberry extract with overlapping confidence intervals, the cranberry extract exhibited higher mean cell viability than blackcurrant in BV-2 cells exposed to K3. This suggests that the antioxidant properties of NAC and blackcurrant extract may not be contributing to cell

survival in BV-2 cells in the same way as in HL-1 cells. Our findings are in agreement with a previous study that showed that cranberry extracts enriched with anthocyanins improved BV-2 cell viability (83). The present findings indicate that some berry extracts may be more effective than others in preserving cell viability depending upon the cell type.

Although the present study focused on the antioxidant properties of berry extracts, the results suggest that the cellular protection of a berry extract may not necessarily translate into its antioxidant potential. For example, cranberries are commonly associated with improvement in urinary tract infections(46). Cranberry extract typically has higher amounts of proanthocyanidins (PAC) in comparison to blackcurrant with a higher anthocyanin phenolic composition (16; 28; 40). Known benefits of PACs include their anti-adherence of bacteria and anti-microbial properties, which inhibit urinary tract infections, inflammation, and periodontal bacteria (16; 40; 46). The anti-inflammatory effects may be a mechanism for the observed cellular protection exhibited by cranberry compared to blackcurrant extract in BV-2 cells. Further studies are needed to explore the biochemical basis of the different effects of the extracts on different cell types.

Taken together, the present study provides evidence that blackcurrant has cardioprotective properties in cardiomyocytes and cranberry extracts may have neuroprotective properties in microglial cells by affecting cell viability. The observation that the antioxidant activity of the extracts was different between the two different cell types was a surprising finding. Furthermore, our cell viability studies may also indicate that depending on the cell type, certain berry extracts may be more effective than others

at cellular protection depending on the berry extract phenotype and cellular mechanisms of action (28).

COMPARISON OF EXTRACT COMPONENTS ON OXIDATIVE STRESS ON BV-2 CELLS

Prior studies of berry extracts such as tart cherry, grape seed, lingonberry, and American ginseng berry have shown that flavonoids reduce oxidative stress *in vitro* (57; 92; 125; 130). Berry flavonoids are composed of key ingredients such as D3R and C3G, which have been associated with both cardioprotective and neuroprotective benefits. D3R, in particular, possesses antioxidant properties, reduces intracellular ROS, and induces bovine ciliary smooth muscle relaxation (84; 85). C3G has been found to suppress ROS and protect endothelial vascular cells from inflammatory-related damage. It has also been associated with gastroprotective and anti-platelet properties (108; 132). Understanding the beneficial properties of the key dominant active compounds as well as the whole berry extract effects on cell models provides important information and may be applicable for further human studies.

The major component of cranberry extract is C3G, whereas the predominant compound present in blackcurrant extract is D3R. In the present study, C3G was modestly more effective at reducing oxidative stress than D3R, consistent with the increased potency of cranberry extract. This suggests that the active component is C3G in cranberry extract.

It is not clear why C3G and D3R have different effects on oxidative stress in cardiomyocytes and microglial cells. Both have been shown to serve as antioxidants in previous studies (31; 85; 108). Given that blackcurrant was a more effective antioxidant in HL-1 cells than cranberry extract, it was hypothesized that the results would be similar

in BV-2 cells. Since the antioxidant activity of the extracts differed between the two cell types, more assays examining various pathways are warranted to further investigate the differences. However, it can be speculated that the origin of oxidative stress in the two different cell types may vary, which leads to different levels of effectiveness for the berry extracts. The possible cellular pathways are discussed below.

EFFECTS OF BERRY EXTRACTS ON NO IN LPS-INDUCED BV-2 CELLS

The resident immune cells in the central nervous system are microglial. Microglia have four functions which include surveillance, phagocytosis, neuroprotection, and toxicity (22). When stimulated with lipopolysaccharide (LPS), microglial cells undergo and induce nitric oxide synthase (iNOS), an enzyme that increases the production of nitric oxide [NO] (3; 22; 137). At low levels NO is neuroprotective, however, at high levels, it can be damaging (77). There was a distinct difference between the blackcurrant and cranberry extracts in their effect on iNOS production. Blackcurrant extract appeared to sensitize or potentiate the cells to LPS-induction of iNOS, whereas cranberry extract inhibited iNOS induction. This finding may be attributed to the nature of the berries. Furthermore, other variables should be considered for this disparate observation between these two berry extracts. Factors that may account for the individual berry effects include how they were cultivated as well as their growth environment- such as temperature conditions that may affect their anthocyanin synthesis. Phenolic content that enhances plant defenses may also be increased in cranberry compared to blackcurrant extract. Thus, cranberry's reported higher inhibition of host responses and bacterial static effects

may impact the reduction in iNOS expression that was observed in the present study with BV-2 cells (9; 16; 42; 133; 138). Additionally, specific genetic factors in berries may also contribute to differences in behaviors. For example, in a recent study by Mengist et al, 128 blueberry varieties were analyzed using trait correlation analysis. They found that underlying genetic contributors may play a role in anthocyanin synthesis. Also, factors such as sugar moiety and fruit size may provide complexity to the diversity of fruit differentiation (95). This study provides an appealing argument as a forward strategy in developing a framework for better investigating bioactive metabolites and fruit quality. However, other methods are commonly used to profile or identify phytochemicals including high-performance liquid chromatography (HPLC) and metabolic profiling. These techniques also have limitations, and conceivably more knowledge can be extracted on berry behavior by understanding their genetic phenotype. Future investigations on the genetics of berries may enhance breeding programs and provide a greater understanding of berry variability and properties. Moreover, they may contribute to our wider understanding of the genus of berry compositions.

Another contributing factor to the effects of berry extracts is L-arginine. L-Arginine is an essential amino acid and precursor for NO (90; 114); NOS catalyzes the oxidation of L-arginine to NO and L-citrulline. L-arginine is a known competitor of NO (114), and this may also be another reason why decreased iNOS expression was observed in the experiments with no effect of NO with cranberry extract. In contrast, blackcurrant extract had greater expression of iNOS and NO production. This is consistent with the reported vasodilator properties of blackcurrant through its principal ingredient D3R. The potentiated effect of blackcurrant on the expression of iNOS and NO may point to the

microglia's ability in boosting neurogenesis (22). This is coupled with blackcurrant's antioxidant effect that was observed in the present study. Blackcurrant may also have neuroprotective benefits. In a review article that summarized evidence on berry extracts and their effects on downregulation of microglia activation, several factors were postulated to be involved through assay measures. *In vitro* and *in vivo* studies demonstrated that flavonoids act on inflammatory pathways such as interleukins, c-Jun-N-terminal kinase, and MAPK pathways important to cellular signaling of proliferation, survival, and apoptosis. These studies provide evidence for berry extracts' effects on dampening immune overactivation of microglia stimulation (60).

Moreover, previous studies of the effects of oxidative stress on iNOS induction in BV-2 cells have been contradictory. In one study, it was reported that inhibition of oxidative stress with NAC markedly increased iNOS expression induced by LPS (137). In another study, iNOS expression was reduced if superoxide dismutase (an enzyme neutralizer to break down harmful oxygen-free radicals in our bodies) was delivered to cells to reduce oxidative stress (73). Therefore, it is not clear whether the antioxidant properties of blackcurrant extract sensitized the cells to LPS induction of iNOS or why cranberry extract inhibited iNOS induction. This is an interesting finding that has implications for brain health. At low levels, NO may serve a neuroprotective role, but at higher levels, NO induces cell death (77). The observation that blackcurrant extract was unable to preserve cell viability during oxidative stress, may reflect its stimulation of iNOS and/or NO production.

It was expected that an increase in iNOS expression with LPS induction would lead to an increase in NO production for blackcurrant, whereas cranberry extract would

inhibit NO production. However, the highest dose of blackcurrant extract induced the largest increase in NO production relative to the no treatment group whereas cranberry extract did not impair NO production. This was also contradictory to previous results observed by Ma et al. (83). They found that cranberry, strawberry, blueberry, red raspberry, black raspberry, and blackberries significantly inhibited NO production. There are several possible explanations for the different results in the present study. Since the studies were conducted in complete BV-2 media, serum proteins may have interfered with the assay, obscuring the effect of berry extracts on NO production (56). Alternatively, operator pipette variability may have affected the results or perhaps greater than three NO assays tests may need to be performed to increase NO sensitivity detection and reduce variability in outcomes. Regardless, significant differences were observed in the effects of blackcurrant and cranberry extracts on iNOS induction by LPS.

The observation that blackcurrant sensitizes the microglia to LPS and increases iNOS expression is particularly interesting when considering previous studies in the CV system. NO is a potent vasodilator and previous studies have demonstrated that D3R ,the main flavonoid in blackcurrant, , induces vasodilation through inhibition of contraction and smooth muscle relaxation in bovine ciliary muscle (85). This may explain why blackcurrant at the highest concentration produced the greatest NO production in the BV-2 cell line in comparison to other experimental groups. Similarly, another berry fruit, aronia berry, was found to enhance NO synthesis in cultured bovine coronary endothelial cells (149).

Although not directly tested in the present study, it can be speculated that berry extracts that stimulate NO production such as blackcurrant and aronia berry may have

cardioprotective health benefits. Increased NO production is associated with endothelial relaxation and therapeutic benefits in the prevention of CVD. NO exerts its effects in the CV system principally by increasing the activity of guanylate kinase that converts GTP to cGMP (97). The increase in cGMP activates protein kinase G (PKG) which ultimately leads to dephosphorylation of the myosin light chains and the relaxation of vascular smooth muscle. In the CV system, NO is principally derived from eNOS expressed in endothelial cells (160). However, since LPS-induced iNOS induction was potentiated in the presence of blackcurrant in microglial cells in the present study, it may well be that blackcurrant extract increases eNOS expression and NO production in vascular endothelial cells. This would enhance vasodilation as previously demonstrated in a study with human subjects (68).

CONCLUSION

In conclusion, the results of this study demonstrate that blackcurrant and cranberry extracts exert antioxidant properties that may benefit human health. Blackcurrant extract effectively inhibited ROS in HL-1 cardiomyocytes and protected them from cell death. In BV-2 cells, cranberry exerted more potent antioxidant effects and inhibited the production of iNOS. The major antioxidant constituents, C3G and D3R, were both effective in reducing oxidative stress as the berry extracts themselves. Interestingly, C3G was slightly more effective when compared to D3R in reducing oxidative stress in BV-2 cells, which may explain the greater effectiveness of cranberry extract in the cells since C3G is the major constituent of cranberry extract. Taken together, the results indicate that the effects of the extracts are not equal and there is some cell specificity to the response, which is a novel finding that was not anticipated.

To our knowledge, this is the first study to specifically compare blackcurrant and cranberry extract, dose responses, and their dominant bioactive compound ingredients in concert. Moreover, a potentially different cellular pathway of action was identified among berry groups and cell types. These results indicate that cranberry extract may be exerting its effects through alternative biochemical pathways to improve survival.

Additionally, there was a parallel direction of the antioxidant - NAC - in association with blackcurrant in cell viability and antioxidant effect. As for limitations to the study, only two cell types were used. If other cell types were used, such as macrophages or skeletal muscle cells, additional information for cell specificity and the various cellular mechanisms at play may have been uncovered. Additional assays are warranted for future research to measure other mediators such as IL-6, IL-8, glutathione, L-arginine, and to determine the antioxidant regulation of cell apoptosis. Further investigations are also needed to determine whether berry extracts exert similar effects in the human body to improve overall health. Research on flavonoid delivery systems that considers bioavailability through modifications of encapsulation formulations and updated dispersion techniques of natural flavonoids is also important in this future area of science (69). Factors such as the use of berry flavonoids as dietary supplements in military healthcare are paramount. Potential nutritional strategies that impact cellular mechanisms involved in CV and neurological diseases may inform practical strategies to improve Warfighter health. By understanding these cellular effects, specific nutritional interventions, dose concentrations, and targeted treatment can be further developed and advanced to help the Warfighter in garrison and theater to mitigate overproduction of oxidative stress contributing to chronic conditions of CVD and neurological diseases. A

conceptual framework is characterized in the figure of the Pillars of Nutritional Readiness with all the many factors that may influence Warfighter health and readiness.

Overall, the intriguing findings in this study indicate that further cell culture studies are needed to understand some of the interesting underlying cellular mechanisms identified in these investigations. Human studies should be considered in the Warfighter population given they are consistently faced with situations yielding high oxidative stress in austere environments of rapid deployment missions and their stressful occupations. Further research is also warranted to test the berry extract's effects on bioavailability and to identify its potential CV and brain health benefits.

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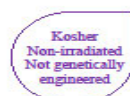
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Appendix A: Berry Extracts



Technical Data Sheet Standardized Black Currant Powder, 30% (*Ribes nigrum*)



Product Code: 24140500
Date: April 2020

Product Specification

Product Description: Pure black currant (*Ribes nigrum*) extract; processed with ethanol as an extract solvent and spray-dried; standardized to a minimum anthocyanin content of 30%

Product Properties

Color	Dark purple
Appearance	Powder, uniform particles, hygroscopic
Taste	Characteristic of black currant; astringent
Odor	Characteristic of black currant; astringent
Mesh size	≥ 95% through 100 mesh (≤ 149 µm)
Excipient	None
Extract ratio	60-80:1
Identity	HPLC anthocyanin profile unique to black currant
Plant part used	Berries (whole fruit)

Analytical Values

		Spec.
Anthocyanins (Expr. as Delphinidin-3-rutinoside chloride; HPLC)	≥ 30.0	g/100 g
Moisture	≤ 5	%
pH	3.0 – 3.5	

Heavy Metals and Pesticides

		Spec.
Lead	< 3	ppm
Cadmium	< 2	ppm
Mercury	< 0.5	ppm
Arsenic	< 1	ppm

Microbiological Values

		Spec.
Yeast	≤ 50	CFU/g
Molds	≤ 50	CFU/g
Total plate count	≤ 1,000	CFU/g
<i>E. coli</i>	Absent	CFU/g
<i>Salmonella</i>	Absent	CFU/10 g

Country of Origin: China (manufacturing), New Zealand (raw materials)

Packaging: Packed in aluminum bag in corrugate box of 5 or 20 kg net

Storage: The product must be stored in a dark, cool, and dry area. Keep the package well-sealed since the product is hygroscopic. Color may change at the end of shelf life.

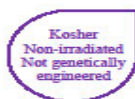
Best Used By: 36 months after production date under proper storage conditions



The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

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Appendix A: Berry Extracts



Technical Data Sheet Standardized Aroniaberry (Chokeberry) Powder, 15% (*Aronia melanocarpa*)



Product Code: 24198000

Product Specification

Product Description: Pure aroniaberry (*Aronia melanocarpa*) extract; processed with water and ethanol as an extract solvent and spray-dried; standardized to a minimum anthocyanin content of 15%

Product Properties

Color	Violet/dark violet
Appearance	Powder, uniform particles, hygroscopic
Taste	Characteristic of aroniaberry; astringent
Odor	Characteristic of aroniaberry
Mesh size (<i>Sieve analysis; 10 min.</i>)	> 98% through 80 mesh (180 µm)
Excipient	None
Solubility	Soluble in water and water/alcohol mixtures
Extract ratio	50-85:1
Identity	Anthocyanin fingerprint comparable to standard
Plant part used	Berries (whole fruit)

Analytical Values

		Spec.
Anthocyanins (<i>Expr. as total anthocyanins; pH-Diff</i>)	≥ 15.0	g/100 g
Polyphenols (<i>Folin; expr. as gallic acid</i>)	≥ 40.0	g/100 g
Moisture (<i>Loss on drying; 1 g; 110° C; 3 hrs.</i>)	≤ 5.0	%
Acidity	7.2 – 13.8	%
Ethanol residue	≤ 5,000	ppm

Heavy Metals and Pesticides

		Spec.
Lead (<i>A.A.</i>)	≤ 1.0	ppm
Arsenic (<i>A.A.</i>)	≤ 1.0	ppm
Cadmium (<i>A.A.</i>)	≤ 0.3	ppm
Mercury (<i>A.A.</i>)	≤ 0.1	ppm

Microbiological Values

		Spec.
Yeast and molds	< 100	CFU/g
Total plate count	< 1,000	CFU/g
Coliforms / <i>E. coli</i>	Absent	/g
<i>Salmonella</i>	Absent	/25 g
<i>Staphylococcus aureus</i>	Absent	/g

Country of Origin: China (manufacturing), Ukraine (raw materials)

Packaging: Packed in aluminum bag in corrugate box of 5 or 20 kg net

Storage: The product must be stored in a dark, cool (10 – 30° C), and dry area. Keep the package well-sealed since the product is hygroscopic. Color may change at the end of shelf life.

Best Used By: 36 months after production date under proper storage conditions

Quality Control

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

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Version Number: 06

Appendix A: Berry Extracts



Certificate of Analysis Standardized Cranberry Powder, 15% PAC (BL-DMAC) (*Vaccinium macrocarpon*) Country of Origin: Canada

Product Code: 54264700
Lot: CN21000280
Production Date: March, 12, 2021
Best by: March 12, 2023

Product Properties

Color	Dark reddish purple
Appearance	Powder, fine, free-flowing
Taste	Characteristic of cranberry
Odor	Characteristic of cranberry
Excipient	None
Extract ratio	300:1
Plant part used	Berries (whole fruit)

Analytical Values

	Value	Spec.
Proanthocyanidins (BL-DMAC)	22.5 g/100 g	≥ 15 g/100 g
Moisture (Loss on drying; 100 – 105° C; 2 hours)	1.37 %	≤ 5 %
Anthocyanins (Expr. as cyanidin-3-glucoside; pH-Diff)	2.13 %	As reported %

Microbiological Values:

	Value	Spec.
Yeast (NF ISO 21527-2)	< 5 CFU/g	< 100 CFU/g
Molds (NF ISO 21527-2)	< 5 CFU/g	< 100 CFU/g
Total plate count (NF EN ISO 4833-1)	< 5 CFU/g	< 1,000 CFU/g
Enterobacteriaceae (NF ISO 21528)	< 5 CFU/g	< 10 CFU/g

Packaging: 5 kg aluminum bag in corrugate box

Storage: The product must be stored in a dark, cool (≤ 75° F/24° C), and dry area. Keep the package well-sealed since the product is hygroscopic. Moisture absorption may cause changes in chemical values.

Best Used By: 36 months after production date under proper storage conditions



Quality Control
Certificate Date: 6/7/21

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

Artemis International, Inc. | 3711 Vanguard Dr. – Ste. A | Fort Wayne, IN 46809-3301 | Tel: (260) 436-6899 | Fax: (260) 478-6900
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Appendix A: Berry Extracts



Technical Data Sheet Standardized Cranberry Powder, 15% PAC (BL-DMAC) (*Vaccinium macrocarpon*)

Product Code: 54264700



Product Specification

Product Description: Pure cranberry (*Vaccinium macrocarpon*) extract; spray-dried and standardized to a minimum proanthocyanidin content of 15% via BL-DMAC

Product Properties

Color	Dark reddish purple
Appearance	Powder, fine, free-flowing, hygroscopic
Taste	Characteristic of cranberry
Odor	Characteristic of cranberry
Excipient	None
Extract ratio	300:1
Plant part used	Berries (whole fruit)

Analytical Values

		Spec.
Proanthocyanidins (BL-DMAC)	≥ 15	g/100 g
Moisture (Loss on drying; 100 – 105° C; 2 hours)	≤ 5	%

Microbiological Values

		Spec.
Yeast	< 100	CFU/g
Molds	< 100	CFU/g
Total plate count	< 1,000	CFU/g
Enterobacteriaceae	< 10	CFU/g

Packaging: 5 kg aluminum bag in corrugate box

Storage: The product must be stored in a dark, cool ($\leq 75^{\circ}\text{F}/24^{\circ}\text{C}$), and dry area. Keep the package well-sealed since the product is hygroscopic. Color may change at the end of shelf life. Moisture absorption may cause changes in chemical values.

Best Used By: 24 months after production date under proper storage conditions

Quality Control

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

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Issue Date: 10/6/20
Version Number: 02

Appendix A: Berry Extracts



Technical Data Sheet ElderCraft® 14% Standardized Elderberry Powder 14% (*Sambucus nigra*)



Product Code: B0120034

Product Specification

Product Description: Pure European black elderberry (*Sambucus nigra*) extract; processed without organic solvent via physical membrane separation and spray-dried with maltodextrin as an excipient; standardized to a minimum anthocyanin content of 14%

Product Properties

Color	Intensely dark red-brown
Appearance	Powder, uniform particles, hygroscopic
Taste	Characteristic of elderberry; astringent
Odor	Characteristic of elderberry
Mesh size	> 98% through 60 mesh (< 250 µm)
Excipient	Maltodextrin (organic, corn-based)
Solubility	Soluble in water and water/alcohol mixtures
Extract ratio	64:1
Identity	HPLC anthocyanin profile unique to elderberry
Plant part used	Berries (fruit)

Analytical Values

		Spec.
Anthocyanins (Expr. as cyanidin-3-glucoside; pH-Diff)	14.00 – 18.00	g/100 g
Anthocyanins (Expr. as cyanidin-3-glucoside; HPLC)	12.50 – 17.50	g/100 g
Polyphenols (Expr. as catechin; Folin-Ciocalteu)	18.00 – 35.00	g/100 g
Color units (E 100%; 510 nm; pH 1.0)	10,000 – 12,000	
Moisture (Loss on drying; 105° C; 30 sec.)	2.0 – 8.0	%
Total acidity (Expr. as citric acid; anhydr.; pH 8.1)	9.00 – 19.00	g/100 g

Heavy Metals and Pesticides

		Spec.
Lead (ICP-MS)	< 0.6	ppm
Arsenic (ICP-MS)	< 0.5	ppm
Cadmium (ICP-MS)	< 1	ppm
Mercury (ICP-MS)	< 0.1	ppm
Pesticides	The processed fruit conforms to the pesticide standards defined in the FDA and EPA regulations.	

Microbiological Values

		Spec.
Yeast (Sabouraud Agar, 28°C, 5 d)	< 20	CFU/g
Molds (Sabouraud Agar, 28°C, 5 d)	< 20	CFU/g
Total plate count (ISO 4833)	< 10,000	CFU/g
<i>E. coli</i> (COLI ID Agar, 37°C, 24 h)	0	CFU/g
<i>Salmonella</i> (ISO 6579)	Absent	/25 g

Country of Origin: Italy (manufacturing), Austria (raw materials)

Packaging: Packed in an aluminum bag in a corrugated box or plastic pail, 5 kg net

Storage: The product must be stored in a dark, cool, and dry area. Keep the package well-sealed since the product is hygroscopic.

Color may change at the end of shelf life.

Best Used By: 36 months after production date under proper storage conditions

Quality Control

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

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Issue Date: 2/5/21
Version Number: 08

Appendix A: Berry Extracts



Technical Data Sheet CherryCraft® 2% (*Prunus cerasus*)



Product Code: B0330070
Date: February 2020

Product Specification

Product Description: Pure European tart cherry (*Prunus cerasus*) extract; processed without organic solvent via physical membrane separation and spray-dried with gum arabic as an excipient; standardized to a minimum anthocyanin content of 2%

Product Properties

Color	Dark red
Appearance	Powder, uniform particles, hygroscopic
Taste	Characteristic of tart cherry
Odor	Characteristic of tart cherry
Mesh size	> 98% through 60 mesh (< 250 µm)
Excipient	Gum arabic
Solubility	Soluble in water and water/alcohol mixtures
Fruit equivalence	50:1
Identity	HPLC anthocyanin profile unique to tart cherry
Plant part used	Berries (fruit)

Analytical Values

	Spec.
Anthocyanins (<i>Expr. as cyanidin-3-glucoside; pH-Diff.</i>)	2.0 – 2.6 g/100 g
Polyphenols (<i>Expr. as catechin; Folin-Ciocalteu</i>)	9.0 – 15.0 g/100 g
Moisture (<i>Loss on drying; 105° C; 30 sec.</i>)	2.0 – 8.0 %
Total acidity (<i>Expr. as citric acid; anhydr.; pH 8.1</i>)	2.00 – 5.00 g/100 g

Heavy Metals and Pesticides

	Spec.
Lead (<i>ICP-MS</i>)	< 3 ppm
Arsenic (<i>ICP-MS</i>)	< 0.5 ppm
Cadmium (<i>ICP-MS</i>)	< 1 ppm
Mercury (<i>ICP-MS</i>)	< 0.1 ppm
Pesticides	Processed fruit conforms to the pesticide standards defined in the FDA and EPA regulations.

Microbiological Values

	Spec.
Yeast (<i>Sabouraud Agar, 28°C, 5 d</i>)	< 20 CFU/g
Molds (<i>Sabouraud Agar, 28°C, 5 d</i>)	< 20 CFU/g
Total plate count (<i>ISO 4833</i>)	< 10,000 CFU/g
<i>E. coli</i> (<i>COLI ID Agar, 37°C, 24 h</i>)	0 CFU/g
<i>Salmonella</i> (<i>ISO 6579</i>)	Absent CFU/25 g

Country of Origin: Italy (manufacturing), Poland (raw materials)

Packaging: Packed in pails of 5 kg net

Storage: The product must be stored in a dark, cool, and dry area. Keep the package well-sealed since the product is hygroscopic.

Color may change at the end of shelf life.

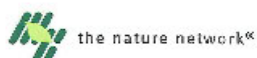
Best Used By: 36 months after production date under proper storage conditions

Quality Control

The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements made are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information, it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product.

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Appendix B: Active Compound COA (Delphinidin 3o-Rutinoside)



PhytoLab

PhytoLab GmbH & Co. KG, Remboldstr. 5-7, 51467 Vöhring, Germany

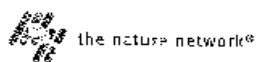
PhytoLab GmbH & Co. KG
Dutendorfer Str. n- /
51467 Vöhring, Germany
Contact:
phytprod® Reference Substances
Tel: +49(0)9163/88-395
Fax: +49(0)9163/88-456
ref-substances@phytoLab.de
Date: 29.04.19

Certificate of analysis

Batch: 78330287
Article: 80735 Delphinidin 3 rutinoside chloride

Test	Unit	Limit	Testresult
Appearance, SOP 100005		powder	Conform
Color, SOP 100006		dark, reddish	Conform
Solubility, SOP 105001: Methanol		soluble	Conform Conform
Identification (UV spectrum from HPL C-11AD analysis) according to specification, SOP 204311		Conform	Conform
Identification (IR-spectroscopy, Ph.Eur. 9.0, 2.2.24)/USP 47 NF 37 <191>], SOP 206000		Conform	Conform
Water content, (micro determination, coulometric titration), Ph.Eur. 9.4., 2.5.32, SOP 304291: Mean value	%		9.4
Chloride (argentometric titration), No. 400897 (double analysis, outsourced): Mean value	%		4.87
Delphinidin 3-rutinoside (HPLC), method 1 (gradient method, 520 nm, % AU), SOP 401278	%	> = 55.00	99.81
Purity, (HPLC), SOP 401367		Conform	Conform
Residual solvents, (headspace-GC), SOP 805765: Residual solvents	%		0.14

Appendix B: Active Compound COA (Cyanidin 3-Glucoside)



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info@phyto-lab.de

Date: 13.09.18

Cust.No: 90776

Certificate of analysis

Report-No.: 61325654 - 99 002
Batch: 13053
Article: 89616 Cyanidin 3-glucoside (supplied as Cl salt)
Quantity: 1 x 25 mg

Test	Unit	Limit	Testresult
Appearance, SOP 100006		powder	Conform
Color, SOP 100006		dark, reddish	Conform
Solubility, SOP 105001, Methanol		soluble	Conform
Identification (UV spectrum from HPLC-DAD analysis) according to specification, SOP 204311		Conform	Conform
Identification (IR spectroscopy, Ph.Eur. 9.9, 2.2.24) (USP 39 NF 34 <197>), SOP 206000		Conform	Conform
Identification (1H-NMR-spectroscopy), (outsourced), SOP 206010		Conform	Conform
Identification (13C-NMR-spectroscopy), (outsourced), SOP 206020		Conform	Conform
Water content, (micro determination, coulometric titration), Ph.Eur. 9.4., 2.5.32, SOP 30479** Mean value	%	≤ 10.0	2.9
Cyanidin 3-glucoside chloride, (HPLC), method 2 (5 AU), SOP 401832	%	≥ 98.00	99.66

Appendix C: Cell Viability Assay



Certificate of Analysis

This document certifies that this product has met all of the quality control standards defined by Cell Signaling Technology, Inc.

Research Use Only Reagent (RUO): For research use only. Not for use in diagnostic procedures.

Product Number: **13837**

Product Name: **Cell Health Assay Kit**

Product Type: Assay Kit

Species of Origin: N/A

Lot Number: 3

Concentration: N/A

Approved Applications:

- Other: Assay Kit

Approval:

Production: Kristen Cullen

Date: 12-September-2017

Appendix D: Measure of Oxidative Stress Assay



CellIROX® Oxidative Stress Reagents

Catalog nos. C10422, C10443, C10444, C10448

Table 1 Contents and storage

Material	Catalog no.	Amount	Excitation/Emission	Concentration	Storage*
CellIROX® Deep Red Reagent	C10422	5 × 50 µL	640/665 nm (Deep Red)	2.5 mM stabilized solution in DMSO	• ≤ -20°C • Protect from light • Desiccate
CellIROX® Orange Reagent	C10443		545/565 (Orange)		
CellIROX® Green Reagent	C10444		485/520 (Green)		
CellIROX® Variety Pack	C10448	1 × 50 µL CellIROX® Deep Red Reagent 1 × 50 µL CellIROX® Orange Reagent 1 × 50 µL CellIROX® Green Reagent			

*When stored as directed, the product is stable for 6 months from the date of receipt.

Introduction

CellIROX® Oxidative Stress Reagents are fluorogenic probes designed to reliably measure reactive oxygen species (ROS) in live cells. The cell-permeable reagents are non-fluorescent or very weakly fluorescent while in a reduced state and upon oxidation exhibit strong fluorogenic signal. CellIROX® Green Reagent is a DNA dye, and upon oxidation, it binds to DNA; thus, its signal is localized primarily in the nucleus and mitochondria. In contrast, the signals from CellIROX® Deep Red and CellIROX® Orange Reagents are localized in the cytoplasm. The fluorescence resulting from CellIROX® Oxidative Stress Reagents can be measured using traditional fluorescence microscopy, high-content imaging and analysis, microplate fluorometry, or flow cytometry. These reagents can be detected using the appropriate benchtop instrument such as the Attune® Acoustic Focusing Cytometer, Tali® Image-based Cytometer, and FLoid™ Cell Imaging Station (See Table 2, page 2).

The staining workflow is simple (Figure 2, page 2), and the reagent can be applied to cells in complete growth medium or buffer. All of the CellIROX® Oxidative Stress Reagents are very photostable when compared to traditional ROS detection dyes. In addition, some of the reagents retain their signal after formaldehyde fixation and detergent permeabilization (Table 2, page 2), allowing for assay flexibility and improved workflows compared to those based on classic dyes for ROS detection.

CellIROX® Oxidative Stress Reagents specifically detect ROS as shown by inhibition of menadione-induced ROS in endothelial cells (Figure 3, page 3). These probes have been used to evaluate ROS generated by various agents, including lipopolysaccharide, menadione, angiotensin II, and nefazodone in several different live-cell models.