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The Antioxidant and DNA Repair Capacities of Resveratrol,

Piceatannol, and Pterostilbene

Justin Ryan Livingston

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

The Antioxidant and DNA Repair Capacities of Resveratrol, Piceatannol, and Pterostilbene

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Lifestyle diseases represent a large burden on developed societies and account for much morbidity worldwide. Research has shown that eating a diet rich in fruit and vegetables helps to ameliorate and prevent some of these diseases. Antioxidants found in fruits and vegetables may provide a substantial benefit in reducing disease incidence. This thesis examines the antioxidant properties of resveratrol, piceatannol, and pterostilbene, and the ability of Burkitt's Lymphoma (Raji) cells to uptake these three antioxidants. It also studies the effect of the antioxidants in protecting against DNA damage and their role in DNA repair following oxygen radical exposure in Raji cells. The Oxygen Radical Absorbance Capacity (ORAC) assay was used to measure overall antioxidant contribution as well as the ability of Raji cells to uptake antioxidant following exposure to 2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH). The single cell gel electrophoresis (Comet) assay was used to assess DNA damage and DNA repair rates of cells. Results showed that Raji cells, following oxygen radical exposure, significantly uptake pterostilbene ($p < 0.0001$), but not piceatannol or resveratrol. Piceatannol provided protection against hydrogen peroxide induced DNA damage, but pterostilbene and resveratrol increased DNA damage following hydrogen peroxide treatment. None of the compounds showed any effect on DNA repair. Overall, this study indicates there is merit for further research into the bioactive roles, including antioxidant capacity, of all three compounds. Such research may provide evidence for the more widespread use of these and other food based compounds for preventing lifestyle diseases.

Keywords: antioxidant, comet assay, DNA repair, ORAC, piceatannol, pterostilbene, resveratrol

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TABLE OF CONTENTS

TITLE PAGE.....	i
ABSTRACT	ii
ACKNOWLEDGEMENTS.....	iii
TABLE OF CONTENTS	iv
LIST OF FIGURES	vi
INTRODUCTION	1
MATERIALS AND METHODS.....	18
Chemicals.....	18
Materials	18
Equipment	18
Compound Preparation	18
Procedure	19
Oxygen Radical Absorbance Capacity (ORAC) Assay.....	19
Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure.....	20
Direct DNA Damage by Compounds	20
Protection against DNA Damage.....	20
DNA Repair.....	20

Comet Assay.....	21
Statistics	21
RESULTS.....	23
ORAC Antioxidant Values	23
Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure.....	23
Lysate Fraction	23
Membrane Fraction.....	24
DNA Damage Caused by Raw Compounds	25
DNA Protection.....	26
DNA Repair Assay	27
DISCUSSION.....	29
Analysis of Antioxidant Abilities.....	29
DNA Protection Analysis	30
DNA Repair Analysis	32
CONCLUSION.....	33
REFERENCES	34
APPENDIX A: Manuscript Accepted for publication in <i>Journal of Food Research</i>	40
APPENDIX B: Manuscript Submitted to <i>The Bryologist</i>	50
APPENDIX C: Presentations	73

LIST OF FIGURES

Figure 1 Mortality in the United States, 2013	1
Figure 2 Glycolysis Pathway	3
Figure 3 Citric Acid Cycle.	4
Figure 4 Electron Transport Chain	5
Figure 5 Reactive Oxygen Species	6
Figure 6 Generation of Hydroxyl Radical Mediated DNA Damage	7
Figure 7 ROS Generation and Removal.....	8
Figure 8 The Antioxidant Response Element (ARE).....	10
Figure 9 Oxidation States of Lipoic Acid	13
Figure 10 Structures of Resveratrol, Pterostilbene, and Piceatannol.....	15
Figure 11 Antioxidant Values of Pterostilbene, Piceatannol, and Resveratrol	23
Figure 12 Mean Antioxidant Values of Raji Cell Lysates	24
Figure 13 Mean Antioxidant Values of Raji Cell Membranes	25
Figure 14 Tail Moments of Raji Cells Following Treatment with Various Compounds.....	26
Figure 15 DNA Protection Assay	27
Figure 16 DNA Repair Assay for Pterostilbene	27
Figure 17 DNA Repair Assay for Piceatannol	28
Figure 18 DNA Repair Assay for Resveratrol.....	28

INTRODUCTION

Lifestyle diseases are diseases that are mainly the result of poor daily habits or of an “inappropriate” relationship of people with their environment (Sharma & Majumdar, 2009). Some of the main risk factors that contribute to these diseases include poor diet, physical inactivity, and harmful behaviors such as smoking and alcohol abuse. The most common lifestyle diseases include heart disease, cancer, stroke, and diabetes. Lifestyle diseases are responsible for the majority of mortality in the United States (Figure 1); however, many of these diseases are preventable (Kochanek, 2014). The behaviors associated with these diseases often result in a lifestyle and metabolic imbalance leading to chronic inflammation. Studies have

Data Brief 178: Mortality in the United States, 2013

Data table for Figure 3. Number of deaths, percentage of total deaths, and age-adjusted death rates for the 10 leading causes of death in 2013: United States, 2012–2013

Rank ¹	Cause of death [based on <i>International Classification of Diseases, Tenth Revision (ICD–10)</i>]	2013			2012		
		Number	Percent of total deaths	Age-adjusted death rate ²	Number	Percent of total deaths	Age-adjusted death rate ²
...	All causes	2,596,993	100.0	731.9	2,543,279	100.0	732.8
1	Diseases of heart (I00–I09,I11,I13,I20–I51)	611,105	23.5	169.8	599,711	23.6	170.5
2	Malignant neoplasms (C00–C97)	584,881	22.5	163.2	582,623	22.9	166.5
3	Chronic lower respiratory diseases (J40–J47)	149,205	5.7	42.1	143,489	5.6	41.5
4	Accidents (unintentional injuries) (V01–X59,Y85–Y86)	130,557	5.0	39.4	127,792	5.0	39.1
5	Cerebrovascular diseases (I60–I69)	128,978	5.0	36.2	128,546	5.1	36.9
6	Alzheimer’s disease (G30)	84,767	3.3	23.5	83,637	3.3	23.8
7	Diabetes mellitus (E10–E14)	75,578	2.9	21.2	73,932	2.9	21.2
8	Influenza and pneumonia (J09–J18)	56,979	2.2	15.9	50,636	2.0	14.4
9	Nephritis, nephrotic syndrome and nephrosis (N00–N07,N17–N19,N25–N27)	47,112	1.8	13.2	45,622	1.8	13.1
10	Intentional self-harm (suicide) (*U03,X60–X84,Y87.0)	41,149	1.6	12.6	40,600	1.6	12.6
...	All other causes (residual)	686,682	26.4	...	666,691	26.2	...

... Category not applicable.

¹Indicates code is not included in ICD–10.

²Based on number of deaths.

³Deaths per 100,000 U.S. standard population.

SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 1 Mortality in the United States, 2013. Mortality in the United States broken down by the 10 leading causes of death. The top 2 killers are heart disease and cancer representing together 46% of all deaths. Overall, lifestyle diseases represent the majority of causes of death. Taken from NCHS data brief 178 (Kochanek, 2014).

shown that a balanced diet rich in fruits, vegetables, and whole grains, helps to reduce the occurrence of these diseases (Bagchi et al., 2003; Mullen et al., 2002; Salucci, Stivala, Maiani, Bugianesi, & Vannini, 2002). One of the main components of fruits and vegetables that is believed to play a major role in the prevention of these diseases are antioxidants.

Antioxidants are molecules that prevent the inappropriate oxidation of other molecules. Inappropriate oxidation occurs when a particular molecule, called an oxidizing agent or oxidant, steals electrons from another molecule, often causing it to have an unpaired electron and leading to the formation of a free radical. This sets off a dangerous chain reaction that can damage many important biomolecules such as DNA. Oxygen is a very common oxidizing agent and one that is crucial for many biological systems, including many in humans. Normal human metabolism involves the breakdown of glucose through a process called glycolysis (Figure 2).

Glycolysis involves a series of phosphorylations, isomerizations and de-phosphorylations involving the starting compound glucose. A variety of enzymes are used in this process which ultimately leads to the net production of two NADH, two pyruvates, and two ATP molecules. Following glycolysis, through a series of steps mediated by the enzyme pyruvate dehydrogenase, two more NADH molecules are produced as the two pyruvate molecules are converted to two acetyl-CoA molecules. The two acetyl-CoA molecules are later used as substrates in the Citric Acid Cycle. The Citric Acid Cycle involves the reaction of acetyl-CoA with oxaloacetate to produce six NADH, two GTP, and two FADH₂ (Figure 3). These combined products are later used in the electron transport chain to produce the chemical and electrical gradients used by the ATP synthase to produce ATP, the currency of cellular energy (Figure 4).

As part of the electron transport chain, electrons are passed from the NADH molecules through complex I. Iron-Sulfur (Fe-S) centers in complex I shuttle the electrons to ubiquinone

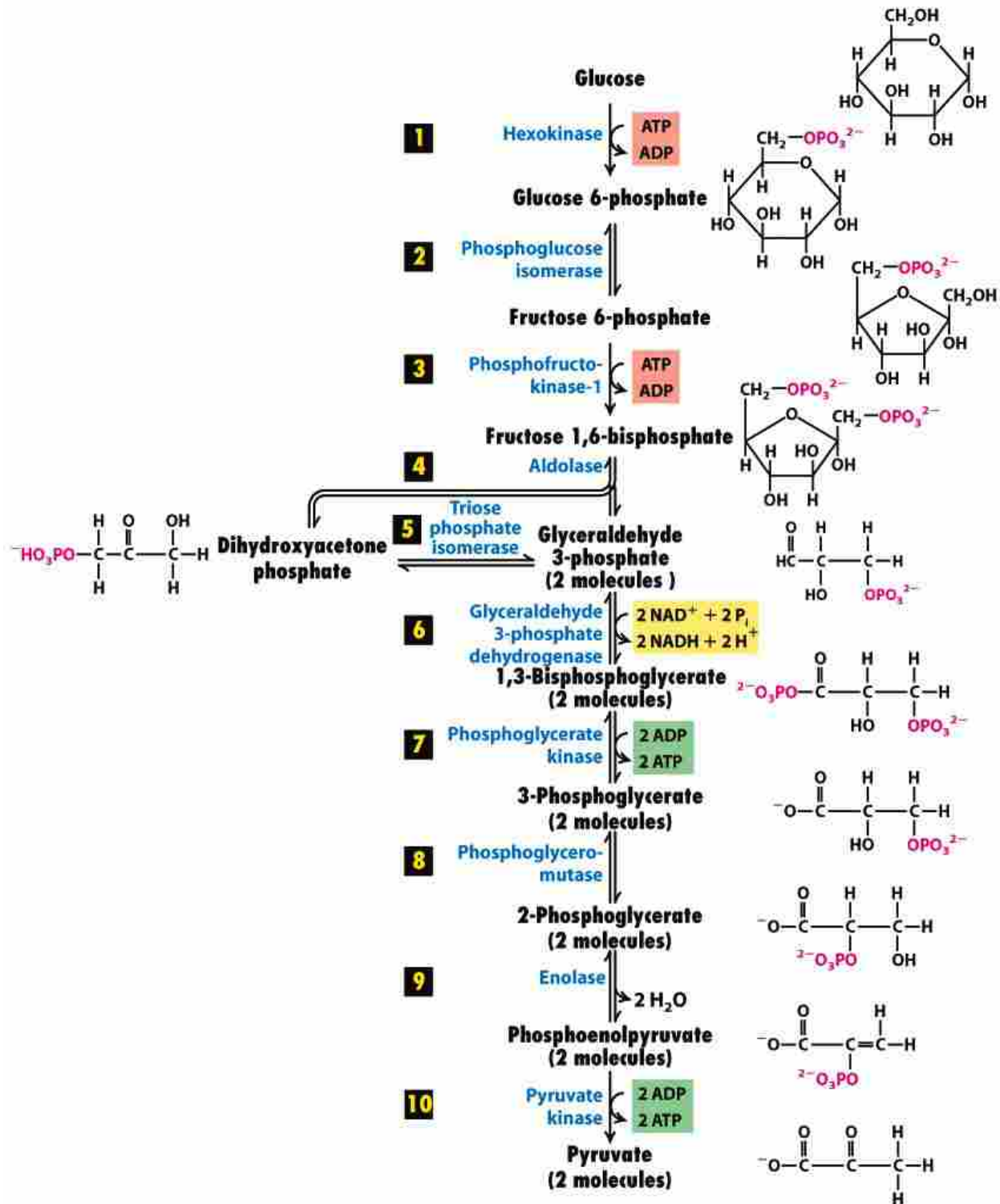


Figure 2 Glycolysis Pathway. Glycolysis a series of reactions that catabolizes glucose into pyruvate. The inputs for glycolysis include glucose, 2 ATP, and 2 NAD⁺. The resulting net products are 2 ATP, 2 NADH and 2 pyruvates. The resulting products are used in humans as part of the Citric Acid cycle and Electron Transport Chain ultimately culminating in the production of ATP by ATP synthase. Taken from Molecular Cell Biology 7E pg. 521.

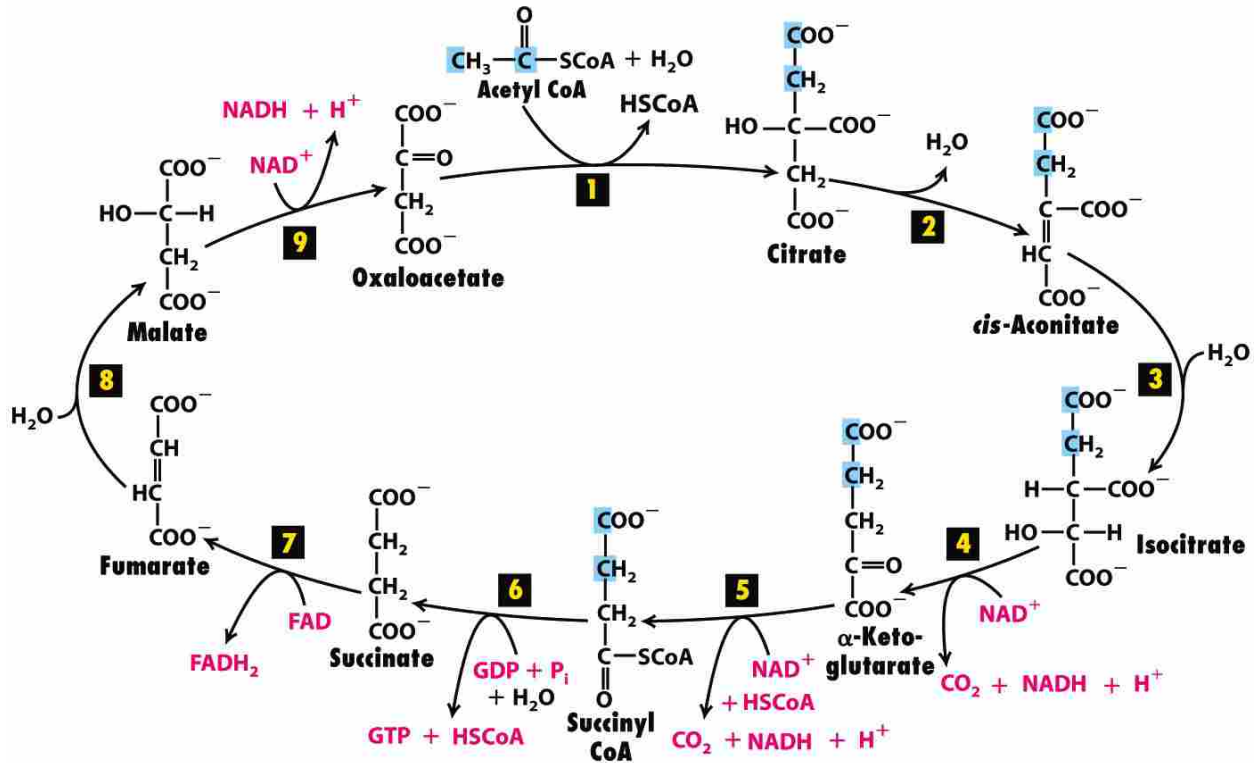


Figure 3 Citric Acid Cycle. The citric acid cycle involves the conversion of 2 acetyl CoA into 4 CO₂, 6 NADH, 2 FADH₂, and 2 GTP. The NADH and FADH₂ produced are used as electron donors in the electron transport chain. Taken from Molecular Cell Biology 7E pg. 528.

which helps them pass to the other complexes of the electron transport chain. FADH₂ follows a similar pattern in complex II. Ubiquinone then donates the electrons to an Fe-S center or to the cytochromes in complex III. Cytochrome C then transfers electrons to complex IV where ultimately these electrons are donated to molecular oxygen, a terminal electron acceptor (Lodish, 2013).

During this whole process of cellular respiration, as much as 4% of the molecular oxygen used leads to the formation of the superoxide anion radical (D. L. Nelson, Cox, Michael M., 2008), (figure 5). This occurs largely in complex I as a result of close contact of molecular oxygen to Fe-S clusters, FMN, dihydroquinone, or through leaking of electrons from semiquinone (Lodish, 2013). The body normally has an enzyme called superoxide dismutase (SOD) which rapidly converts superoxide anion radicals into hydrogen peroxide and molecular

oxygen. Another enzyme called glutathione peroxidase converts hydrogen peroxide into water.

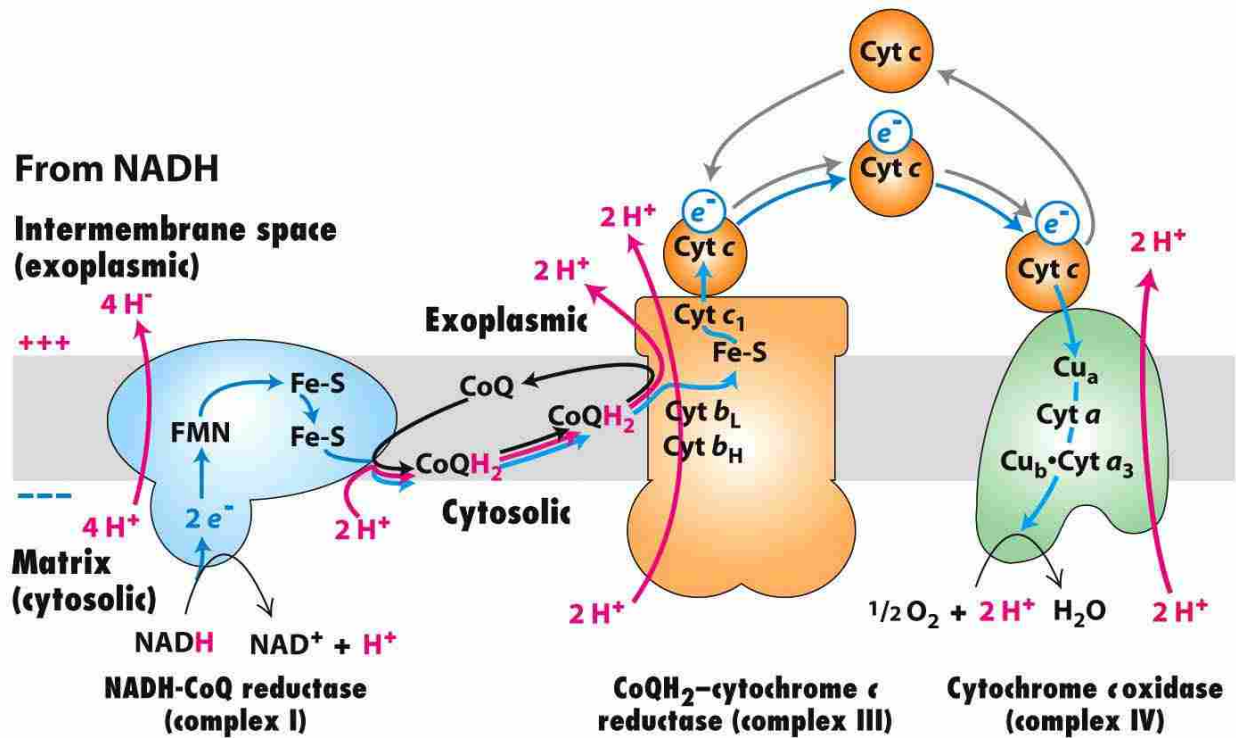
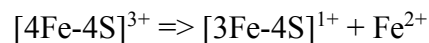
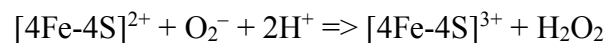


Figure 4 Electron Transport Chain. Electrons are donated by NADH and FADH₂ into complexes I and II. The electrons are passed through FE-S centers to ubiquinone (coenzyme Q) and then shuttled to the cytochromes of complex III. Cytochrome C transfers the electrons to complex IV where oxygen acts as a terminal electron receptor. Protons are pumped from the cytosol into the intermembrane space. This creates the proton motive force used by ATP synthase. Taken from Molecular Cell Biology 7E pg. 536.

Superoxide anion radicals generated during respiration can damage the FE-S centers of complexes I and II, resulting in the release of free iron into the cytosol. This takes place as superoxide anion radicals interact with the Fe-S centers and steal away an electron causing the center to be oxidized and hydrogen peroxide to be produced. This subsequently causes free Fe²⁺ to be released from the center into the cytosol. Superoxide causes damage to Fe-S centers by the following reaction (Imlay, 2003):



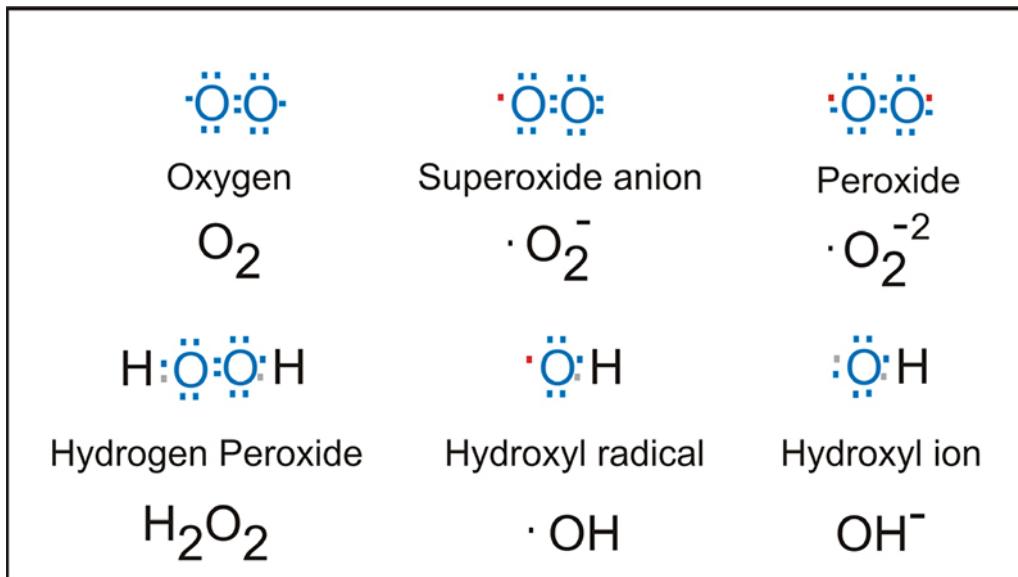
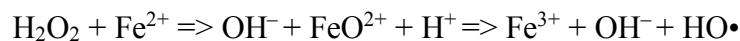


Figure 5 Reactive Oxygen Species. Highly reactive oxygen containing molecules are collectively referred to as reactive oxygen species (ROS). These include superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals. Taken from http://www.biotek.com/assets/tech_resources/10592a/figure1.jpg

Hydrogen peroxide produced by the damage of Fe-S centers, as well as that produced by the action of SOD, is then converted to hydroxyl radicals by the free cytosolic Fe^{2+} via the Fenton reaction. The Fenton reaction involves the creation of hydroxyl radicals from hydrogen peroxide by ferrous (Fe^{2+}) iron. Hydrogen peroxide is reduced to hydroxide as the Fe^{2+} is oxidized to Fe^{3+} and binds to peroxide. The peroxide then binds to a hydrogen ion to produce a hydroxyl radical. The overall reaction is as follows:



The hydroxyl radicals produced by the Fenton reaction are extremely reactive and readily react with DNA and other important biomolecules causing severe damage (Figure 6), (Imlay, 2003). DNA damage can lead to instability and mutations in the genome. Transforming mutations are considered to be a hallmark of cancer (Hanahan & Weinberg, 2011).



Figure 6 Generation of Hydroxyl Radical Mediated DNA Damage. Molecular oxygen interacts with complexes I or II of the electron transport chain and steals electrons from the Fe-S centers producing superoxide anion radicals. Superoxide anion radicals interact with the Fe-S centers, damage them, and cause release of free Fe²⁺ into the cytosol. Hydrogen peroxide reacts with the free Fe²⁺ to produce hydroxyl radicals which directly damage DNA. Taken from Imlay (2003).

Superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals are highly reactive and toxic. These, as well as other highly reactive oxygen containing compounds, have been collectively called reactive oxygen species (ROS). The body has natural means of quickly removing these compounds through enzymes such superoxide dismutase (SOD), glutathione

reductase and catalase. Failure to properly remove these ROS leads to DNA, lipid, and protein damage (Figure 7), (Imlay, 2003; Lodish, 2013).

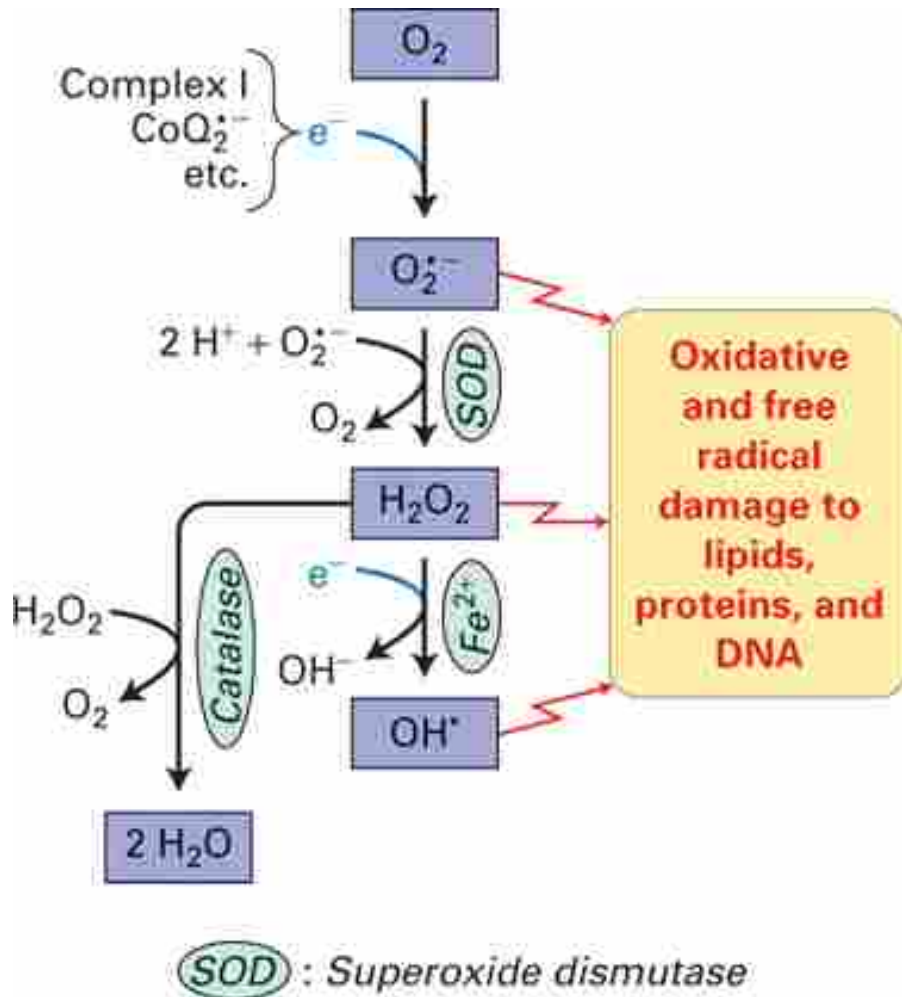


Figure 7 ROS Generation and Removal. Molecular oxygen steals electrons from complexes I and II of the electron transport chain producing superoxide anion radicals. Superoxide anion radicals are converted by superoxide dismutase (SOD) into hydrogen peroxide. Catalase or glutathione peroxidase converts hydrogen peroxide into water. ROS are very toxic and, if not removed, can damage DNA, lipids, and proteins.

Normal cellular processes are estimated to produce about 2×10^{10} superoxide and hydrogen peroxide species per cell, per day (Ji, Dickman, Kang, & Koenig, 2010). However, ROS can also be induced as a result of exposure to ultraviolet light, radiation, and smoke (Forman, Fukuto, & Torres, 2004). The increase of ROS increases the potential to cause

oxidative injury to macromolecules such as nucleic acids, proteins and lipids. Cellular damage caused by ROS can lead to necrosis and ATP depletion, which can prevent apoptosis, one of the “symptoms” of cancer (Lee & Shacter, 1999). Oxidative stress has also been linked to other lifestyle diseases such as atherosclerosis, and diabetes (Spector, 2000). Exercise can also dramatically elevate the level of ROS in the body (Childs, Jacobs, Kaminski, Halliwell, & Leeuwenburgh, 2001). During exercise, mitochondria in skeletal muscle produce nitric oxide synthase (NOS), an enzyme that produces an intermediate of nitric oxide (NO^\cdot), a highly reactive molecule. NO^\cdot and O_2 (in radical form) can react to produce peroxynitrite, ONOO^\cdot , which is a powerful oxidant that can break the ROS cellular equilibrium and can lead to DNA injury. Physical activity can also activate leukocyte infiltration, which may lead to inflammation due to ROS release. Leukocytes, especially phagocytes, are large producers of ROS that kill bacteria and foreign antigens but can also cause tissue damage. Research has shown that there is an increase of neutrophil infiltration into skeletal muscle after strenuous physical activity, which elevates normal levels of ROS in the body (Leeuwenburgh & Heinecke, 2001). Research also confirms higher ROS levels in humans after downhill running (Childs et al., 2001; Hilbert, Sforzo, & Swensen, 2003; MacIntyre, Reid, Lyster, & McKenzie, 2000).

Cells, however, have an antioxidant defense system that alleviates the effects of oxidative stress by preventing ROS from being formed or by removing or neutralizing them from the cell (Evans & Halliwell, 2001). This defense system, called the Antioxidant Response Element (ARE), is a transcription factor binding site located upstream of the promoter region of antioxidative and cytoprotective genes (Figure 8). A family of transcription factors called NRF2 activates the ARE. NRF2 is usually bound to Keap 1, a cytoplasmic protein (Nguyen, Nioi, & Pickett, 2009). Under normal conditions, Keap1 induces the ubiquitination of NRF2, which has

a half-life of 20 minutes. Under oxidative stress, cysteine residues in Keap1 are disrupted and NRF2 is released. NRF2 migrates to the nucleus, where it binds to the ARE and induces the transcription of several antioxidant-producing enzymes (Wang et al., 2007). Cells can also uptake antioxidants from their environment through passive and active transport when needed (Honzel et al., 2008).

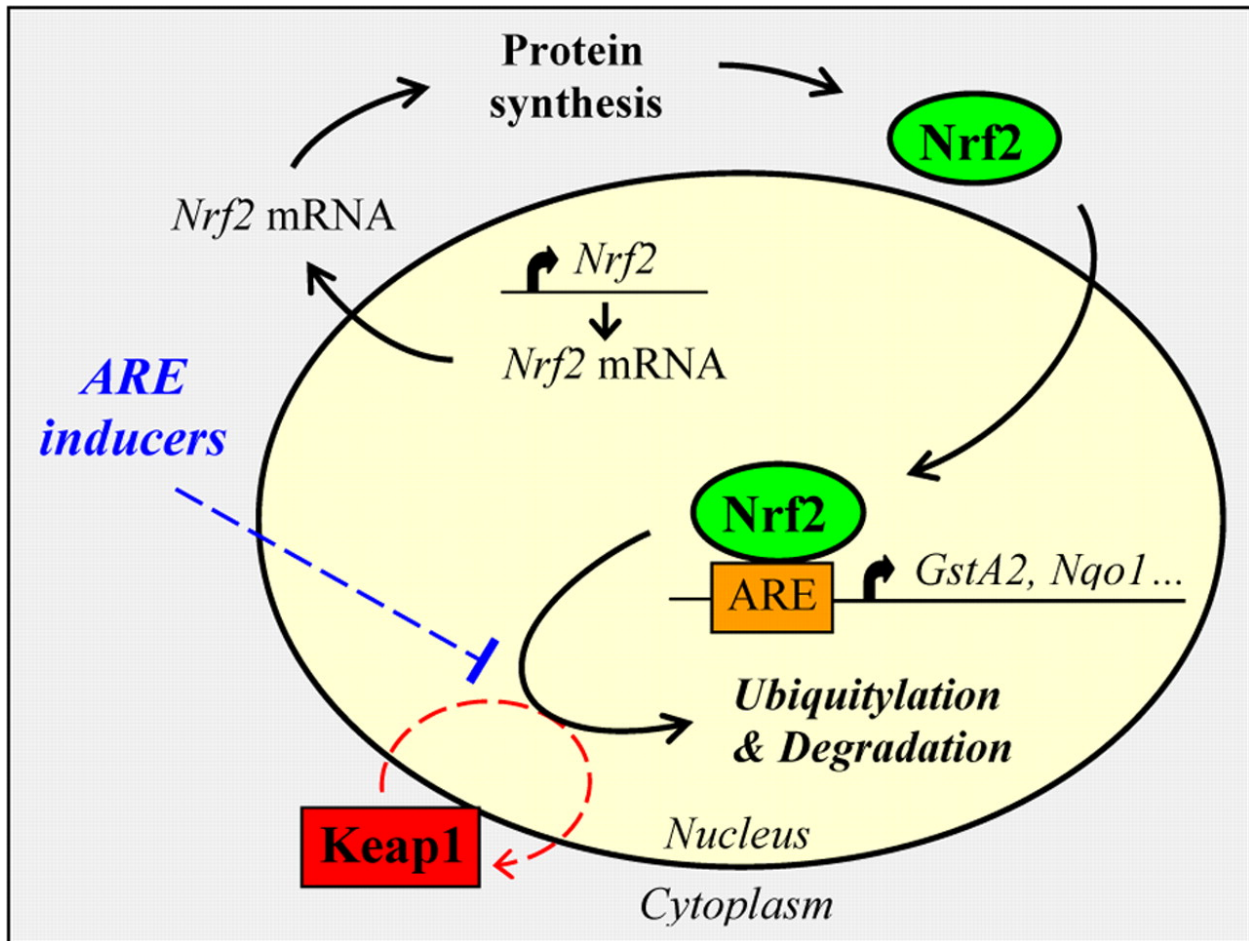


Figure 8 The Antioxidant Response Element (ARE). Nrf2 is a transcription factor that is bound by Keap1 in the cytosol. Keap1 normally causes Nrf2 to be ubiquitinated and degraded. Following oxidative stress, Keap1 releases Nrf2 and Nrf2 migrates to the nucleus where it acts to transcribe several important ARE genes.

Oxidative stress takes place when the concentration of reactive oxygen species (ROS) exceeds cellular antioxidant capacity (Adly, 2010). In order to maintain cellular equilibrium, ROS must be produced and consumed at approximately equal rates (Gupta et al., 2012).

Environmental factors such as smoking, poor diet and obesity, alcohol, radiation, carcinogens,

etc can trigger oxidative stress. Chronic systemic oxidative imbalance is believed to be one of the major causes of lifestyle diseases such as cardiovascular disease, ischemic stroke, diabetes and cancer (Mahalingaiah & Singh, 2014; Singh, Vrishni, Singh, Rahman, & Kakkar, 2010).

It has been estimated that an unhealthy diet coupled with physical inactivity account for 10% of the global burden of disease (England, Andrews, Jago, & Thompson, 2015). Unhealthy dietary habits include a low consumption of fruits and vegetables. Recent research has shown that a diet with high fruit and vegetable consumption may reduce the risk of lifestyle and other diseases (Bussel et al., 2015; Carter, Gray, Troughton, Khunti, & Davies, 2010; Epps, 2013; Joshipura et al., 1999).

Lifestyle diseases are a large area of concern and the subject of intensive research globally (Edwards et al., 2014). Our bodies naturally produce oxygen radicals as part of our cellular metabolism, but our cells also possess mechanisms to deal with those stresses (A. R. Garrett et al., 2014). These natural mechanisms provide considerable protection, however, excess oxygen radicals and reduced antioxidant availability may lead to imbalance and disease (Koppenhofer et al., 2015). Oxidative imbalance due to lifestyle factors can be partially prevented by consuming antioxidants.

Substances such as L-ascorbic acid (vitamin C), α -Tocopherol (vitamin E), resveratrol, glutathione, and lipoic acid serve as antioxidant sources against oxidative stress and therefore are thought to have chemopreventive properties. L-ascorbic acid (vitamin C) has proven to be one of the most successful chemopreventive compounds for non-hormone-dependent cancers. Vitamin C is a water-soluble compound that is absorbed by cells through passive and active transport (active transport accounts for the greater portion of vitamin C accumulation in cells) (Wilson, 2005). In humans, vitamin C must be acquired from diet due to lack of gulonolactone

oxidase, an enzyme that synthesizes L-ascorbic acid (Rumsey & Levine, 1998). Studies show that higher ingestion of vitamin C has a high correlation with lower risk for pancreatic, cervical, and colorectal cancer (Block, 1991). Also, higher intake of vitamin C during pregnancy decreases risk of brain tumors during childhood. Vitamin C also aids in the regeneration of vitamin E and other antioxidants (Block, 1991).

Vitamin E, a lipophilic antioxidant, refers to a group of tocopherols and tocotrienols that have also shown chemopreventive properties. Mostly absorbed through active transport using lipoprotein channels, vitamin E protects cell membranes from lipid peroxidation and quenches ROS when fat is oxidized (Chan, 1993; Ju et al., 2010). The most abundant form of vitamin E in cells is α -tocopherol, but even though it has the highest antioxidant activity of all vitamin E forms, δ -tocopherol and λ -tocopherol are more effective at free radical quenching and therefore are considered the types of vitamin E that account for cancer preventive properties (Yang, Suh, & Kong, 2012) (Yang, Suh, & Kong, 2012).

Glutathione is the major soluble endogenous antioxidant found in cells. It is a simple compound made of glutamate, cysteine, and glycine. Its role is to scavenge free radicals and peroxides. Glutathione or its precursors are absorbed by active transport, using gamma-glutamyltransferase, or passive transport, which is dependent on sodium ions (Hagen, Aw, & Jones, 1988; Ortega, Mena, & Estrela, 2011). Glutathione is usually found in its reduced form and is rapidly consumed when oxidants are produced, thus maintaining cellular equilibrium (Balendiran, Dabur, & Fraser, 2004). Glutathione has several other functions including the regeneration of vitamin C and vitamin E, DNA synthesis and repair, and it is often involved in cell proliferation and cell death (Ortega et al., 2011). Under oxidative stress, normal cells respond by upregulating the production of glutathione. In cancer cells, glutathione production is

less regulated. Some cancer cell lines exhibit glutathione depletion and other cancer cell lines such as bone marrow, breast, lung, larynx, and colon have shown increased levels of glutathione. This helps to protect them from the harmful effects of chemotherapy drugs (Balendiran et al., 2004; Ortega et al., 2011).

Lipoic acid, called the “universal antioxidant”, is a small amphipathic molecule with two thiol groups that can be oxidized or reduced (Goraca et al., 2011). The reduced form of lipoic acid, or dihydrolipoic acid (DHLA), is the major form of lipoic acid that reacts with ROS, however, the oxidized form can also inactivate free radicals (Figure 9), (Roberts & Moreau, 2015).

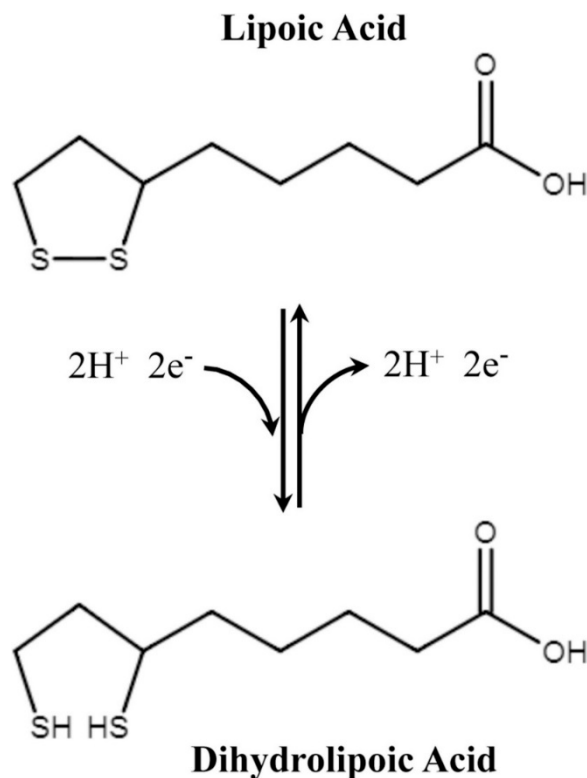


Figure 9 Oxidation States of Lipoic Acid. Lipoic Acid is a potent antioxidant that can be found intracellularly in either its oxidized (Lipoic Acid) or reduced (Dihydrolipoic Acid) forms. These molecules play a large role in intracellular redox balance. Taken from Roberts & Moreau (2015)

Both forms of lipoic acid are able to regenerate vitamin C and vitamin E. Lipoic acid is mostly obtained from diet, but cells can synthesize it in small amounts if needed. Because of its amphipathic properties, lipoic acid can easily cross biological membranes and protect the cell from oxidants in every cell compartment (Goraca et al., 2011). Lipoic acid has been shown to protect cells from gamma radiation in mice (Ramachandran & Nair, 2011), and to induce apoptosis in murine melanoma cells, ovarian epithelial cancer cells, and breast cancer cells without inducing apoptosis in normal non-transformed cells (Na, Seo, & Kim, 2009; Pack, Hardy, Madigan, & Hunt, 2002; Vig-Varga et al., 2006).

Antioxidants from diet, such as those found in fruits and vegetables, reduce oxidative stress and prevent oxygen radical DNA damage (Kuate, 2013; Sagrillo et al., 2015). Many fruits and vegetables have been shown to exhibit robust antioxidant activity (Gupta-Elera et al., 2012; Gupta-Elera, Garrett, Martinez, Robison, & O'Neill, 2011). One antioxidant, resveratrol, has previously been reported to have high antioxidant activity and was believed to be a central factor in what is known as the French Paradox (Aschemann-Witzel & Grunert, 2015; Yamagata, Tagami, & Yamori, 2015). The French Paradox is the observation that even though the French diet includes large amounts of saturated fats, they have a low incidence of coronary heart disease. Their high intake of red wine was believed, among other things, to be responsible for the lower disease incidence rates because it contains the powerful antioxidant resveratrol.

Resveratrol can be found naturally in many food sources such as grapes, peanuts, and berries (Calamini et al., 2010; Rimando, Kalt, Magee, Dewey, & Ballington, 2004). There has been evidence suggesting that the *in vivo* effect of resveratrol may be limited due to its low bioavailability (Davidov-Pardo & McClements, 2015; Semba et al., 2014). Bioavailability is the ability of a drug to enter the circulation and cause an effect once it is consumed.

Resveratrol is a phytochemical produced by plants in response to a fungal infection (Jang et al., 1997). Resveratrol has been found to be a preventive compound for several pathologies such as cardiovascular disease, viral infections, Alzheimer's disease, and cancer (Li, Gong, Dong, & Shi, 2012; Ruan, Lu, Song, & Zhu, 2012; Xie et al., 2012). Additionally it has been shown to stop tumor initiation, promotion, and progression (Jang et al., 1997; Jannin et al., 2004). Resveratrol has the ability to quench ROS such as superoxide and hydroxyl radicals. It especially protects the mitochondria, an organelle that produces high concentrations of ROS (Kitada, Kume, Imaizumi, & Koya, 2011). Resveratrol can be absorbed by cells through passive transport, but it can also be absorbed by carrier-mediated transport as it associates with several serum proteins to enter the cells. This helps to facilitate resveratrol absorption by cells (Jannin et al., 2004).

Resveratrol has two relatively lesser studied analogs, pterostilbene and piceatannol, which may provide a valuable alternative to resveratrol for protection against chronic disease. Piceatannol structurally differs from resveratrol by an additional 3' hydroxyl group (Figure 10), which may contribute to better antioxidant and bioavailability properties than resveratrol (A. R. Garrett et al., 2014; Tang & Chan, 2014). Piceatannol can be naturally found in berries, grapes, and passion fruit (Maruki-Uchida et al., 2013; Rimando et al., 2004). Pterostilbene is a dimethyl ether form of resveratrol (Figure 10), and is also found naturally in grapes and blueberries (Sato

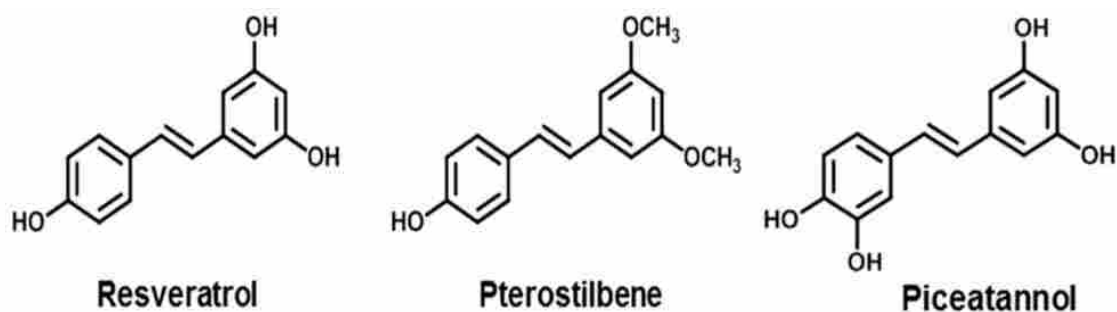


Figure 10 Structures of Resveratrol, Pterostilbene, and Piceatannol.

et al., 2014). Pterostilbene has also been shown to have greater bioavailability than resveratrol and may serve as a more attractive alternative to resveratrol for antioxidant and other functional properties (Kapetanovic, Muzzio, Huang, Thompson, & McCormick, 2011). Overall these three compounds have been reported to exhibit a variety of potential biological effects such as anticancer effects (Xia, Deng, Guo, & Li, 2010), cardiovascular disease prevention (McCormack & McFadden, 2013), and apoptosis induction (Jancinova, Perecko, Nosal, Svitekova, & Drabikova, 2013).

The purpose of this thesis was: (1) to compare the antioxidant properties of resveratrol, pterostilbene, and piceatannol, (2) to assess the ability of Burkitt's Lymphoma (Raji) cells to uptake these three compounds following challenge with an oxidizing stimulus, (3) to determine the ability of these compounds to protect against DNA damage, and (4) to identify the effect of resveratrol, piceatannol, and pterostilbene on DNA repair rates in Raji cells. We used the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the overall antioxidant properties as well as the ability of cells to uptake antioxidant following exposure to AAPH and the single cell gel electrophoresis (Comet) assay to measure DNA protection and DNA repair.

The ORAC assay is a well-established method that is widely accepted and used in the food industry. It is used as a measure of the antioxidant capacity of foods, vitamins and phytochemicals alike (Andrew R. Garrett, Murray, Robison, & O'Neill, 2010). The ORAC assay measures the ability of an antioxidant to protect against the oxidative degradation of the fluorescent molecule fluorescein by the oxygen radical generator AAPH. The intensity of the fluorescence diminishes over time as fluorescein becomes increasingly oxidized and this decay is recorded over a two hour period. The antioxidant being tested is compared to a standard antioxidant, Trolox, a water soluble vitamin E analog. The lesser the degradation of fluorescein

that takes place over the 2 hour period, the stronger the antioxidant. The decay curve of the antioxidant treated fluorescein is compared to the decay curve of the Trolox treated fluorescein to produce an antioxidant rating.

The single cell gel electrophoresis (comet) assay is a widely used and sensitive method for detecting and quantifying DNA damage (Olive, Banath, & Durand, 2012). It involves treating cells with a DNA damaging agent and then embedding the cells in low melting point agarose on microscope slides. The cells are lysed and then electrophoresed to separate out the DNA fragments into “comets” for fluorescence staining and fluorescence microscopy imaging. The amount of damage can be assessed by detecting the length of the DNA fragmentation of these comets as well as the intensity of the fluorescence of different parts of the comets.

Due to the structural differences discussed earlier, we hypothesized that pterostilbene and piceatannol would exhibit higher utilization and protective effect compared to resveratrol following oxidative challenge. We also hypothesized that these compounds would provide protection against DNA damage and modulate DNA repair when compared to a placebo phosphate buffered saline (PBS) treatment.

MATERIALS AND METHODS

Chemicals

2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH), Fluorescein sodium salt, Propidium Iodide, Resveratrol, and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). Piceatannol and Pterostilbene were purchased from Cayman Chemical (Ann Arbor, MI). Hydrogen Peroxide was purchased from Fisher Scientific (Pittsburg, PA).

Materials

Costar 3694 96 well plates were obtained from Corning Inc. Cellstar 12 well cell culture plates were obtained from Greiner Bio-One International.

Equipment

A BMG FLUOstar Optima plate reader (S/N 413-0225) was used to measure fluorescence readings for ORAC assays. A Zeiss Axioscope fluorescence microscope was used to image all Comet experiments. A Misonix Sonicator 3000 was used for cell lysis.

2.4 Cell Culture

Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations at 37°C and 5% CO₂.

Compound Preparation

Resveratrol, pterostilbene, and piceatannol were each dissolved initially at 40mM concentrations in DMSO. These samples were then diluted in PBS to 400 μM aliquots. These aliquots were further diluted, in PBS, as needed to working concentrations for their respective assays. Aliquots were stored in fluorescence protection bags at -20°C until needed. Aliquots

were tested before freezing and after being frozen to ensure effectiveness; no difference in effectiveness was observed.

Procedure

Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were diluted to a 24 μM concentration and then analyzed according to the method published in Gupta-Elera et al. (2012) with the following modifications: 20 μL of AAPH was used instead of 25 μL AAPH, and assays were run for 120 minutes instead of 90 minutes. The final concentration of all samples tested was 2 μM . The ORAC assay was used because it is a well-established method of measuring antioxidant activity *in vitro* (Andrew R. Garrett et al., 2010). The sample to be tested was mixed with a fluorescent molecule (Fluorescein) and an oxygen radical generator (AAPH). The level of fluorescence was measured every 2 minutes over a 120 minute period to create fluorescence decay curves. Experimental samples were run in the same plate as a standard (Trolox) and control samples (Fluorescein alone and Fluorescein plus AAPH). Samples were run in a BMG FLUOstar Optima plate reader with 485 nm emission and 590 nm excitation filters. Samples were run in six different plates with twelve replicates per plate for each compound.

Standard Curve

The area under the curve (AUC) was measured for all experimental and standard samples from their respective fluorescence decay curves. The AUC for each sample and standard was calculated by subtracting the AUC of Fluorescein plus AAPH from the AUC of the sample/standard. The AUC for the standard samples were then plotted to create a standard curve. The AUC of each sample was compared to the standard curve to determine its antioxidant activity in Trolox Equivalent per mg sample (TE/mg).

Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure

Cellular uptake of antioxidant by Raji cells after oxygen radical generator exposure was measured by following the methods published in Gupta-Elera et al. (2011) with some modifications. In short, Raji cells were incubated with AAPH for 10 min to simulate oxidative stress. Following incubation, they were washed and then treated with 10 μ M antioxidant for 10, 20, 45, or 60 min. Following treatment, the samples were washed 2 times and sonicated to lyse. Following sonication, the samples were centrifuged for 30 min at 3000 g. The supernatant was then removed as the lysate fraction. The pellet was washed and used as the membrane fraction. Three different plates per compound were run for both membrane and lysate fractions, with twenty-four replicates per plate.

Direct DNA Damage by Compounds

Raji cells were incubated with either hydrogen peroxide, PBS, or one of the three compounds for 90 min at a 10 μ M concentration. The cells were then washed and prepared for comet assay as described below.

Protection against DNA Damage

Raji cells were incubated for 90 min at a 10 μ M concentration of the compounds. Following incubation, cells were washed twice and then exposed to 10 μ M H₂O₂ for 10 min. The cells were then washed and re-suspended in warm RPMI 1640 and prepared for comet assay as described below.

DNA Repair

Raji cells were incubated in 500 μ L of 10 μ M H₂O₂ for 10 minutes to induce DNA damage. Following incubation, 1 mL of cold RPMI 1640 media was added to the cells. The cells were then centrifuged at 450 g for 5 min at 4°C, washed with cold PBS, and re-suspended in

fresh warm media. Cells were then treated with the compounds and allowed to repair for a predetermined amount of time (0, 5, 15, 30, 60, and 90 min). Samples were then prepared for comet assay as described below.

Comet Assay

Following the damage, protection, and repair protocols, samples were prepared for comet analysis by following the methods described by Xiao et al. (2014) with slight modifications. Briefly, samples were mixed with low melting point agarose and layered on double frosted microscope slides (Xiao et al., 2014). The slides were placed in alkaline lysis buffer for 60 min, rinsed with ddH₂O and then placed in alkaline electrophoresis buffer for 20 minutes. They were then electrophoresed for 30 min. Following electrophoresis, slides were allowed to rest in ddH₂O for 15 min, then fixed in -20°C 100% ethanol for 5 min and allowed to dry prior to being stained with propidium iodide and imaged. All comets were scored using TriTek CometScore Freeware v1.5. Samples were run in three different trials with fifty comets imaged per sample, totaling 150 comets per sample.

Comet Assay results are reported in terms of Tail Moment. Tail moment is defined as the product of the tail length and the % of DNA in the tail. These values are given as part of the output by the CometScore software and are widely reported for Comet analysis (Olive et al., 2012).

Statistics

All statistics were performed using JMP Pro 11. ANOVA and the Tukey-Kramer HSD tests were used to compare compounds and PBS for all ORAC and cellular uptake data. Welch's T test was used to compare treatment and non-treatment groups for the comet repair data. A Dunnett's Procedure was used to compare all treatment groups with the PBS treatment for the

DNA damage and protection data. All statistics were calculated at the family-wise $\alpha=0.05$ level.

All error bars represent the 95% confidence interval of the mean.

RESULTS

ORAC Antioxidant Values

The raw antioxidant values of pterostilbene, piceatannol, and resveratrol as measured by ORAC are given in Figure 11. Resveratrol exhibited the highest antioxidant activity with a mean value 311.403 TE/mg followed by piceatannol and pterostilbene with means of 283.520 TE/mg and 126.875 TE/mg, respectively. There was a significant difference ($p < 0.0001$) in the mean TE/mg values among the tested compounds.

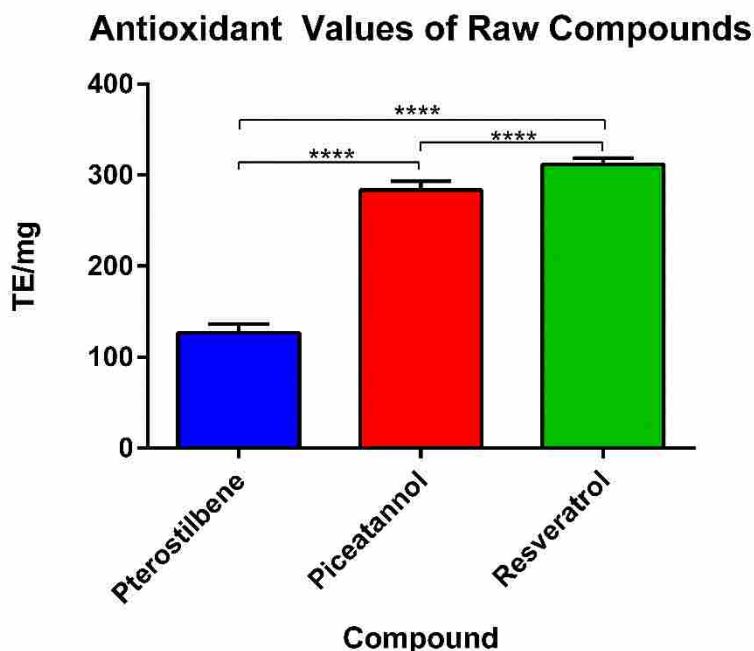


Figure 11 Antioxidant Values of Pterostilbene, Piceatannol, and Resveratrol. Values, measured by ORAC assay, are reported as mean Trolox Equivalents per milligram (TE/mg). The mean for each compound was significantly different than those of the other two compounds ($p < 0.0001$, $n = 72$).

Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure

Lysate Fraction

Figure 12 shows the results from our cellular uptake model for the lysate fraction of Raji cells. There was no significant difference in antioxidant levels measured across the different time points or different concentrations (1 and 10 μM) of the compounds (data not shown). However

there was a significant difference between the compounds. Treatment of Raji cells with pterostilbene had the highest antioxidant activity with a mean of 22,407 TE/L/10⁶ cells followed by piceatannol and resveratrol with mean values of 16,319 TE/L/10⁶ cells and 12,424 TE/L/10⁶ cells, respectively.

Treatment of Raji cells with pterostilbene produced significantly higher antioxidant values ($p < 0.0001$), compared to piceatannol, resveratrol and the control. We also found significantly higher antioxidant values in Raji cells treated with piceatannol compared to those treated with resveratrol ($p = 0.0283$).

Antioxidant Capacity Cellular Model Lysate Fraction

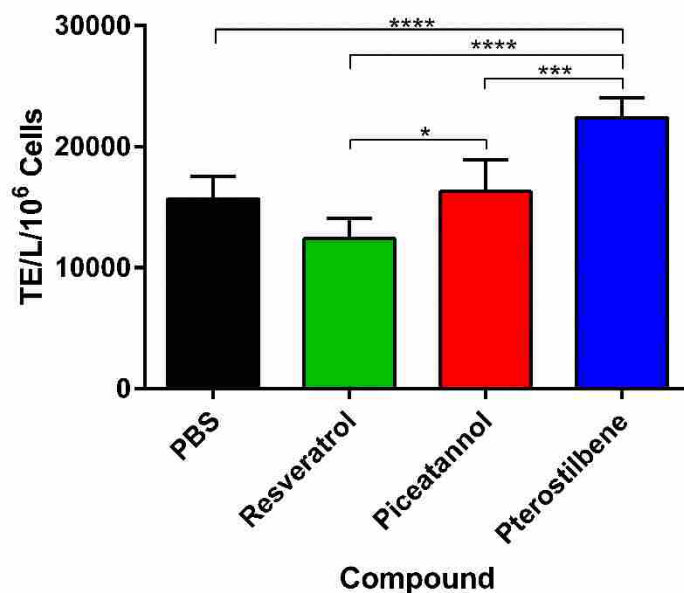


Figure 12 Mean Antioxidant Values of Raji Cell Lysates. Antioxidant values of cell lysate following challenge with AAPH and recovery with the specified antioxidant treatments. Values reported as Trolox Equivalents per Liter per 10⁶ cells (TE/L/10⁶ cells). $n = 72$ for each treatment.

Membrane Fraction

We found no significant difference in antioxidant levels measured across the different time points or different concentrations (data not shown). Contrary to the lysate, the mean antioxidant values for all three compounds were less than raw Trolox (standard), (Fig 13). The

treatment with the highest antioxidant value was pterostilbene, (mean = -4614 TE/L/10⁶ cells). This treatment was the only treatment that was significantly different from the other groups (p < 0.0001). Piceatannol, resveratrol, and PBS had mean antioxidant values of -11024 TE/L/10⁶ cells, -11390 TE/L/10⁶ cells, and -10612 TE/L/10⁶ cells, respectively. Antioxidant values are reported as negative because they are being compared to a standard curve of raw Trolox, as opposed to cells treated with Trolox. Thus, the amount of antioxidant in the membrane fraction is not as high as raw Trolox. There is no precedent for a Trolox standard curve from cells incubated with Trolox so results were compared to a raw Trolox standard curve.

Antioxidant Capacity Cellular Model Membrane Fraction

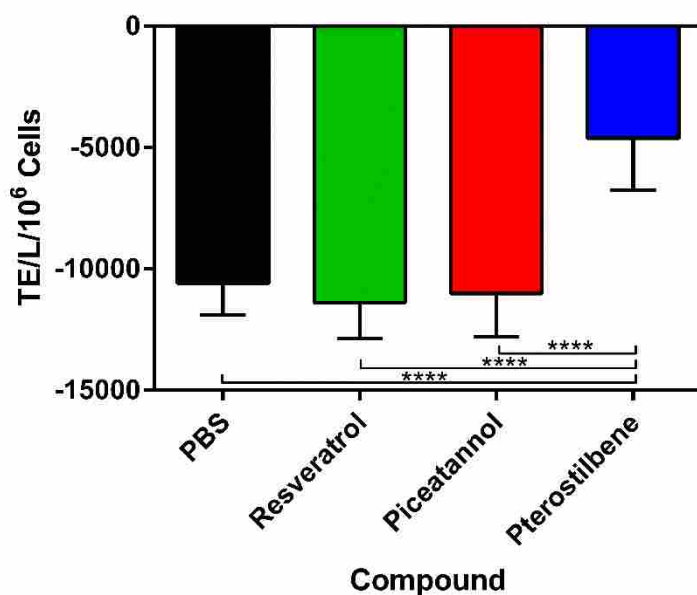


Figure 13 Mean Antioxidant Values of Raji Cell Membranes. Antioxidant values of cell membranes following challenge with AAPH and recovery with the specified antioxidant treatments. Values reported as Trolox Equivalents per Liter per 10⁶ cells (TE/L/10⁶ cells). n = 72 for each treatment.

DNA Damage Caused by Raw Compounds

To ensure that treating the cells didn't cause DNA damage by itself, Raji cells were treated with hydrogen peroxide, PBS, or compound and then analyzed by comet assay. Hydrogen

peroxide caused significantly more damage to Raji cells, as measured by tail moment, than PBS ($p < 0.0001$). No other treatment caused a difference in damage compared to PBS (Figure 14).

DNA Damage from Treatment with Raw Compound

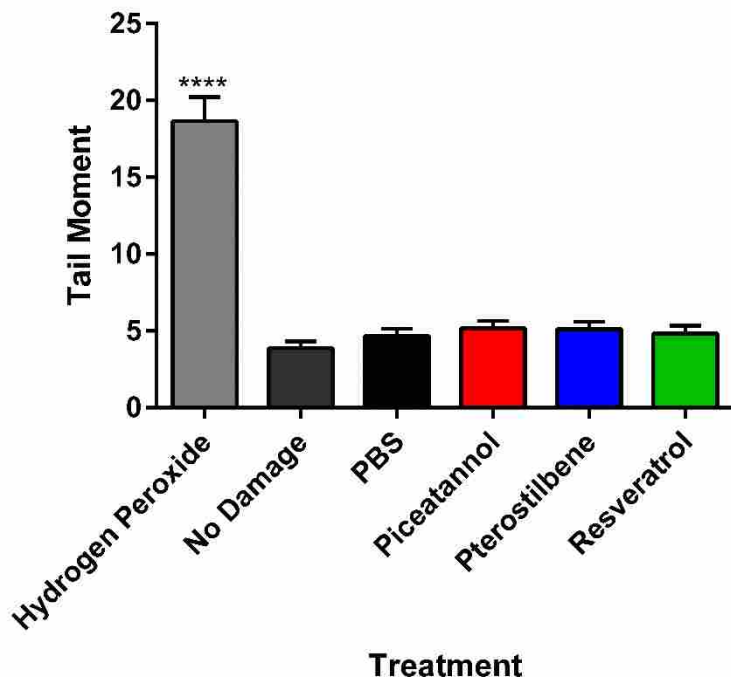


Figure 14 Tail Moments of Raji Cells Following Treatment with Various Compounds. DNA damage caused by the treatments was analyzed by Comet assay. Damage is reported as tail moment (comet tail length \times %DNA in comet tail), where larger tail moments indicate more damage. $n = 150$ samples per treatment.

DNA Protection

The DNA protection assay was performed by pretreating Raji cells with one of the stilbenoid compounds or the control (PBS) prior to treatment with hydrogen peroxide (Figure 15). Surprisingly, samples pretreated with pterostilbene and resveratrol exhibited larger tail moments than samples pretreated with PBS alone, ($p < 0.0001$). Samples pretreated with piceatannol had smaller tail moments than samples pretreated with PBS ($p < 0.0001$). No difference was observed between samples pretreated with PBS and samples receiving no pretreatment.

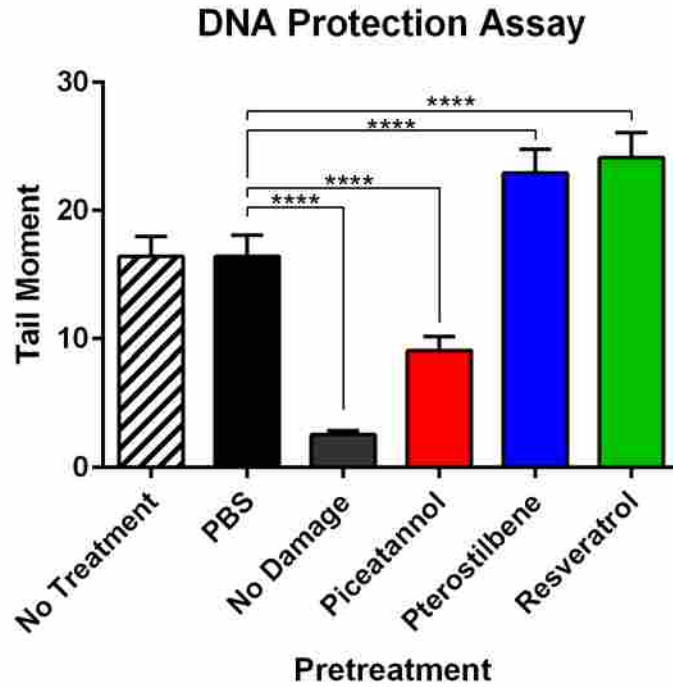


Figure 15 DNA Protection Assay. Raji cells were pretreated with antioxidant compounds prior to treating with hydrogen peroxide. Damage is reported as tail moment. n = 150 for each treatment.

DNA Repair Assay

The results from our Comet repair assay showed a decrease in the tail moment of cells over time (Figures 16, 17, and 18); however, there was no difference in tail moment for samples

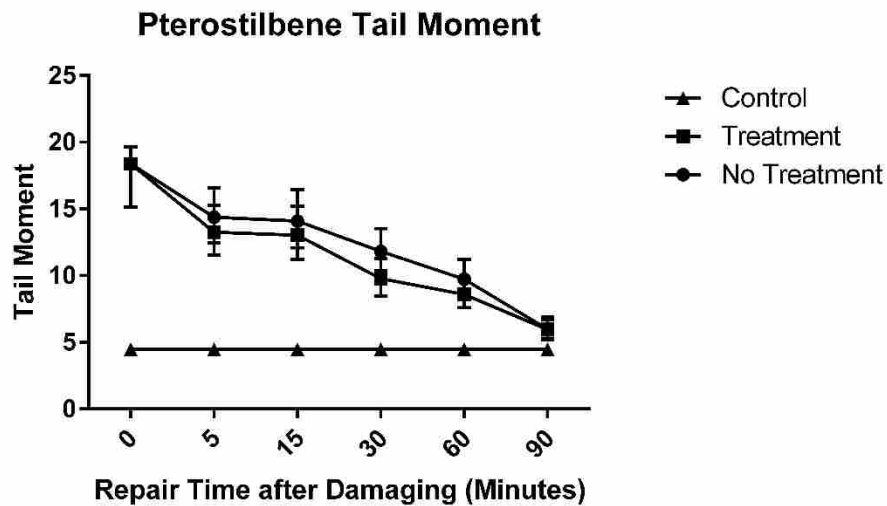


Figure 16 DNA Repair Assay for Pterostilbene. Tail moments were measured for samples treated either with or without pterostilbene over 6 different time points. The differences were insignificant for all time points. n = 150 for each treatment at each time point.

treated with or without pterostilbene, piceatannol, or resveratrol after 90 minutes of repair ($p = 0.9488, 0.7750, \text{ and } 0.6785$ respectively).

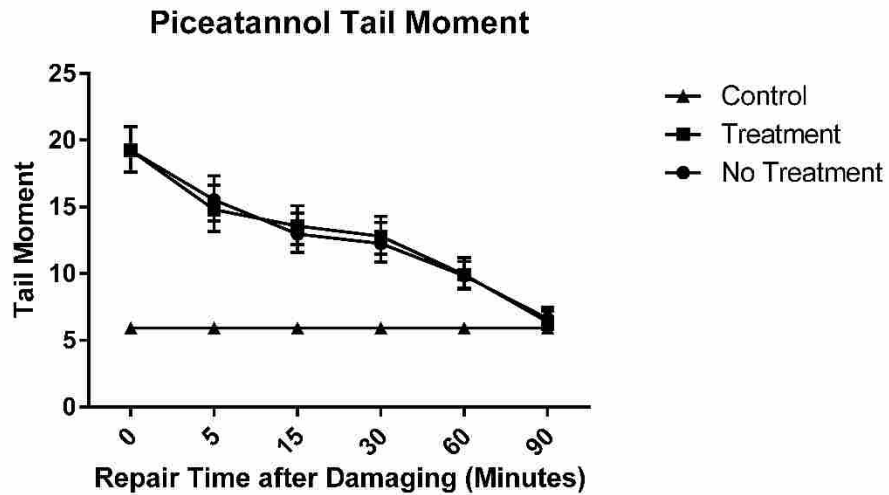


Figure 17 DNA Repair Assay for Piceatannol. Tail moments were measured for samples treated either with or without piceatannol over 6 different time points. The differences were insignificant for all time points. $n = 150$ for each treatment at each time point.

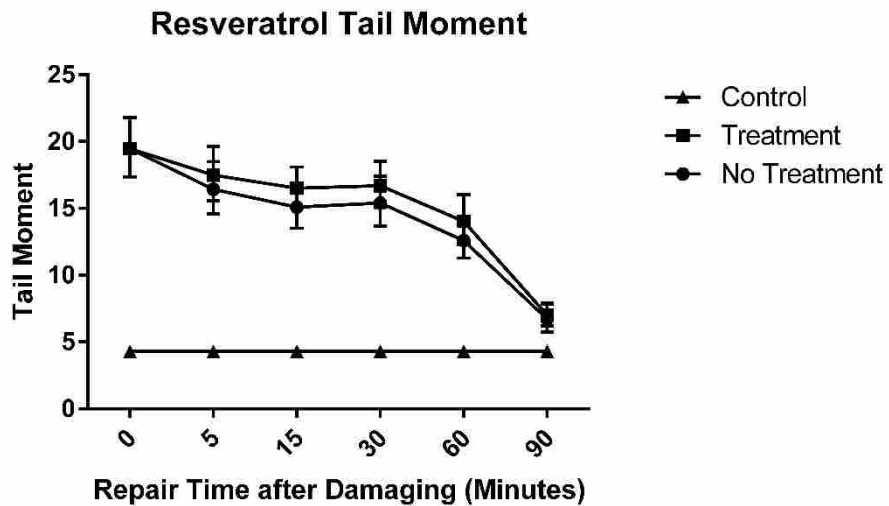


Figure 18 DNA Repair Assay for Resveratrol. Tail moments were measured for samples treated either with or without resveratrol over 6 different time points. The differences were insignificant for all time points. $n = 150$ for each treatment at each time point.

DISCUSSION

The results of this study provided interesting information regarding resveratrol and its analogs: piceatannol and pterostilbene.

Analysis of Antioxidant Abilities

Even though resveratrol demonstrated significantly higher raw antioxidant activity than either piceatannol or pterostilbene, its ability to be absorbed by Raji cells for future antioxidant protection was weak. Pterostilbene is a dimethyl ether form of resveratrol and therefore is more lipophilic than the other two compounds. We hypothesized that it would be incorporated by cells more readily for the means of antioxidant protection than either resveratrol or piceatannol. As suspected, pterostilbene did show a significantly increased ability to be absorbed compared to piceatannol, resveratrol, and control. Piceatannol was also shown to have more absorption ability than resveratrol in Raji cells. We tested this absorptive capacity at different time points, 10, 20, 45, and 60 min, as well as at 1 μ M and 10 μ M, concentrations. ANOVA revealed no significant differences between the concentrations or time points, so the data from all time points and concentrations was combined into 1 group for each compound. These compound groups were then compared to each other for the results reported here. This evidence helps to support our hypothesis that the structures of pterostilbene and piceatannol affect their ability to protect cells from internal oxidizing damage. These results suggest a need for further studies into the biological roles of both pterostilbene and piceatannol.

The ORAC antioxidant values presented in Figure 11 represent the *in vitro* antioxidant ability of all three compounds in the absence of cells. These results have little biological relevance other than to establish the baseline relative antioxidant capacity of all three compounds. From those results we can see that the base antioxidant capacity of resveratrol by

itself is higher than the other two compounds. That information is only moderately useful because it provides no evidence of the antioxidant capacity of resveratrol in cells. The cellular uptake of antioxidant following oxygen radical generator exposure results provide valuable information about the practical antioxidant capacity of these compounds. This is because these antioxidant values are obtained from cells treated with these compounds. The results presented in Figures 12 and 13 provide evidence of the ability of cells to use these compounds to protect against overall oxidizing damage. Thus, even though from Figure 11 we see resveratrol had the highest base antioxidant capacity, its low bioavailability was demonstrated in Figures 12 and 13. We see that piceatannol and even more so, pterostilbene were absorbed more by cells and used to help protect against oxidizing damage. These results provide valuable information about the practical usefulness of these antioxidant in situations of oxidative stress.

DNA Protection Analysis

We investigated the ability of these compounds to protect against DNA damage induced by treatment with hydrogen peroxide. We first wanted to ensure that these compounds were not exerting any cytotoxic effects on the Raji cells, so cells were exposed to each of these compounds. We did not see any loss of viability, compared to cells incubated with PBS, as measured by Trypan blue exclusion after 24, 48, or 72 h, indicating that the results we obtained were not due to loss of cell viability (data not shown). We also wanted to ensure that no DNA damage was induced by these compounds so we performed comet analyses on Raji cells treated with each compound. We observed no difference in tail moments of Raji cells treated with these compounds when compared to cells treated with PBS, indicating that these compounds themselves had not caused DNA damage directly to the cells (Figure 14).

To assess the ability of these compounds to protect Raji cells from DNA damage, we pretreated Raji cells with each compound and then exposed them to hydrogen peroxide. While piceatannol pretreatment demonstrated the ability to protect from DNA damage ($p < 0.0001$), resveratrol and pterostilbene pretreatment resulted in increased DNA damage following hydrogen peroxide exposure, ($p < 0.0001$). The increase in tail moment after pretreatment with resveratrol and pterostilbene may be due to anticancer effects or cell death inducing effects of these agents. We did not observe any loss of viability or increase in DNA damage after treatment with these compounds so pretreatment may predispose Raji cells to undergo cell death following oxidative stress. Xia et al (2010) reported that resveratrol has anticancer effects for prostate, breast and epithelial cancers. These results suggest that this effect may extend to lymphocytic cancers as well. Further work will help to elucidate and characterize these mechanisms.

The results presented in Figures 12 and 13 measure the overall antioxidant capacity of resveratrol, piceatannol, and pterostilbene in cells. In contrast, the DNA protection results presented in Figure 15, measure the effect of the antioxidants for protecting against DNA damage directly. These results come from looking at the physical damage to the DNA detected as both single and double stranded DNA breaks. These results are valuable because they give a better characterization of the intracellular antioxidant effects of these compounds in respect to DNA itself. From these results we saw that piceatannol was able to directly protect against DNA damage. Contrarily, pterostilbene and resveratrol resulted in increased statistically equivalent levels of DNA damage following hydrogen peroxide treatment.

Prior to the protection assay we had incubated the cells with just compound to see if by themselves they resulted in increased DNA. These results showed no evidence of DNA damage caused by the compounds directly. We also cultured the cells with these compounds for 24, 48,

and 72 hours and saw no decrease in viability compared to cells cultured with PBS. Given these two experiments it appears that there is no evident harm to the cells from these compounds. The results of the protection assay therefore may be explained as a synergistic effect of the hydrogen peroxide with pterostilbene or resveratrol in cells. Another explanation is that potentially pterostilbene and resveratrol may result in changes in nucleosome positioning or chromatin structure that result in increased ability of hydrogen peroxide to damage the DNA. The mechanisms for these results need to be further characterized.

DNA Repair Analysis

In an effort to investigate whether or not these compounds could help to repair previously damaged DNA, we tested both a 1 μM (data not shown) and a 10 μM concentration. No positive effects on DNA repair were observed at physiological concentrations. We also looked at several time points to see if there were differences over time. We saw no significant effect on the DNA repair rates of Raji cells that received antioxidant treatment versus those that did not. This result was observed for all time points and concentrations tested.

CONCLUSION

These results indicate that among the many biologically relevant roles of resveratrol, piceatannol, and pterostilbene, DNA repair modulation does not appear to be one of them. This study provided no additional evidence of resveratrol's usefulness as an intracellular antioxidant, but has shown the potential usefulness of pterostilbene in protecting cells from intracellular oxidizing damage. This work also provided information about the possible anticancer activities of all three compounds. This study demonstrates the need to further investigate the biologically active roles of all three compounds. Such work may prove to establish mechanisms which may increase longevity and reduce aging. Foods containing antioxidants should be studied further to provide greater insight into the role of a healthy balanced diet in improving health. Such studies may be valuable in helping to alleviate many lifestyle diseases worldwide.

Future directions for this work include follow up to the observed results of the DNA Protection Assay where we observed increased DNA damage following pretreatment of Raji cells to either resveratrol or pterostilbene and subsequent treatment with hydrogen peroxide. This follow up would involve observing the viability of Raji cells for various time intervals following treatment, to see if they are able to recover from this increased damage. Further work also needs to be conducted to determine if the observed effects are specific to only Raji cells. All of the same experiments need to be conducted on various other cancer cell lines, as well as on human lymphocytes. This work would be important to confirm that resveratrol, piceatannol, and pterostilbene do not have detrimental effects on noncancerous human cells. It would also help to confirm the potential anticancer effects observed in this thesis.

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The Antioxidant and DNA Repair Activities of Resveratrol, Piceatannol, and Pterostilbene

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Abstract

Lifestyle diseases represent a large burden on developed societies and account for much morbidity worldwide. Research has shown that eating a diet rich in fruit and vegetables helps to ameliorate and prevent some of these diseases. Antioxidants found in fruits and vegetables may provide a substantial benefit in reducing disease incidence. This study examines the antioxidant properties of resveratrol, piceatannol, and pterostilbene, and the ability of Burkitt's Lymphoma (Raji) cells to uptake these three antioxidants. It also studies the effect of the antioxidants in protecting against DNA damage, and their role in DNA repair following oxygen radical exposure in Raji cells. The Oxygen Radical Absorbance Capacity (ORAC) assay was used to measure overall antioxidant contribution as well as the ability of Raji cells to uptake antioxidant following exposure to 2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH). The single cell gel electrophoresis (Comet) assay was used to assess DNA damage and DNA repair rates of cells. Results showed that Raji cells, following oxygen radical exposure, significantly uptake pterostilbene ($p < 0.0001$), but not piceatannol or resveratrol. Piceatannol provided protection against hydrogen peroxide induced DNA damage, but pterostilbene and resveratrol increased DNA damage following hydrogen peroxide treatment. None of the compounds showed any effect on DNA repair. Overall, this study indicates there is merit for further research into the bioactive roles, including antioxidant capacity, of all three compounds. Such research may provide evidence for the more widespread use of these and other food based compounds for preventing lifestyle diseases.

Keywords: antioxidant, comet assay, DNA repair, orac, piceatannol, pterostilbene, resveratrol

1. Introduction

It has been estimated that an unhealthy diet coupled with physical inactivity account for 10% of the global burden of disease (England et al., 2015). Unhealthy dietary habits include a low consumption of fruits and vegetables. Cardiovascular disease, ischemic stroke, diabetes, and cancer are some diseases that may develop due to poor lifestyle choices such as diets with low consumption of fruits and vegetables. Recent research has shown that a diet with high fruit and vegetable consumption may reduce the risk of these and other diseases (Bussel et al., 2015; Carter et al., 2010; Epps, 2013; Joshipura et al., 1999).

Lifestyle diseases are a large area of concern and are the subject of intensive research globally (Edwards et al., 2014). Oxidative stress is believed to be one of the major causes of these diseases (Mahalingaiah & Singh, 2014; Singh et al., 2010). Our bodies naturally produce oxygen radicals as part of our cellular metabolism, but our cells also possess mechanisms to deal with those stresses (A. R. Garrett et al., 2014). These natural mechanisms provide considerable protection; however, excess oxygen radicals and reduced antioxidant availability may lead to imbalance and disease (Koppenhofer et al., 2015). Oxidative imbalance due to lifestyle factors can be partially prevented by consuming antioxidants. Antioxidants from diet, such as those found in fruits and vegetables, reduce oxidative stress and prevent oxygen radical DNA damage (Kuate, 2013; Sagrillo et al., 2015). Many fruits and vegetables have been shown to exhibit robust antioxidant activity (Gupta-Elera et al., 2012; Gupta-Elera et al., 2011). One antioxidant, resveratrol, has previously been reported to have high antioxidant activity and was believed to be a central factor in what is known as the French Paradox (Aschemann-Witzel & Grunert, 2015; Yamagata et al., 2015). The French Paradox is the

observation that even though the French diet includes large amounts of saturated fats, they have a low incidence of coronary heart disease. Their high intake of red wine was believed, among other things, to be responsible for the lower disease incidence rates because it contains the powerful antioxidant resveratrol. Resveratrol can be found naturally in many food sources such as grapes, peanuts, and berries (Calamini et al., 2010; Rimando et al., 2004). There has been evidence suggesting that the *in vivo* effect of resveratrol may be limited due to its low bioavailability (Davidov-Pardo & McClements, 2015; Semba et al., 2014). Bioavailability is the ability of a drug to enter the circulation and cause an effect once it is consumed.

Resveratrol has two relatively lesser studied analogs, pterostilbene and piceatannol, which may provide valuable alternatives to resveratrol for protection against chronic disease. Piceatannol structurally differs from resveratrol by an additional 3' hydroxyl group (Figure 1), which may contribute to better antioxidant and bioavailability properties than resveratrol (A. R. Garrett et al., 2014; Tang & Chan, 2014). Piceatannol can be naturally found in berries, grapes, and passion fruit (Maruki-Uchida et al., 2013; Rimando et al., 2004). Pterostilbene is a dimethyl ether form of resveratrol (Figure 1), and is also found naturally in grapes and blueberries (Sato et al., 2014). Pterostilbene has also been shown to have greater bioavailability than resveratrol and may serve as a more attractive alternative to resveratrol for antioxidant and other functional properties (Kapetanovic et al., 2011). Overall these three compounds have been reported to exhibit a variety of potential biological effects such as anticancer properties (Xia et al., 2010), cardiovascular disease prevention (McCormack & McFadden, 2013), and apoptosis induction (Jancinova et al., 2013).

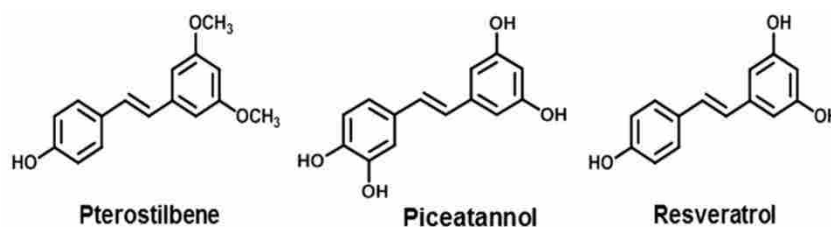


Figure 1. Structures of pterostilbene, piceatannol, and resveratrol

The purpose of this study was: (1) to compare the antioxidant properties of resveratrol, pterostilbene, and piceatannol, (2) to assess the ability of Burkitt's Lymphoma (Raji) cells to uptake these three compounds following challenge with an oxidizing stimulus, (3) to determine the ability of these compounds to protect against DNA damage, and (4) to identify the effect of resveratrol, piceatannol, and pterostilbene on DNA repair rates in Raji cells. We used the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the overall antioxidant properties as well as the ability of cells to uptake antioxidant following exposure to AAPH and the single cell gel electrophoresis (Comet) assay to measure DNA protection and DNA repair. Due to the structural differences discussed earlier, we hypothesized that pterostilbene and piceatannol would exhibit higher utilization and protective effect compared to resveratrol following oxidative challenge. We also hypothesized that these compounds would provide protection against DNA damage and possibly modulate DNA repair when compared to a placebo phosphate buffered saline (PBS) treatment.

2. Materials and Methods

2.1 Chemicals

2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH), Fluorescein sodium salt, Propidium Iodide, Resveratrol, and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). Piceatannol and Pterostilbene were purchased from Cayman Chemical (Ann Arbor, MI). Hydrogen Peroxide was purchased from Fisher Scientific (Pittsburg, PA).

2.2 Materials

Costar 3694 96 well plates were obtained from Corning Inc. Cellstar 12 well cell culture plates were obtained from Greiner Bio-One International.

2.3 Equipment

A BMG FLUOstar Optima plate reader (S/N 413-0225) was used to measure fluorescence readings for ORAC assays.

A Zeiss Axioscope fluorescence microscope was used to image all Comet experiments. A Misonix Sonicator 3000 was used for cell lysis.

2.4 Cell Culture

Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations at 37 °C and 5% CO₂.

2.5 Compound Preparation

Resveratrol, pterostilbene, and piceatannol were each dissolved initially at 40 mM concentrations in DMSO. These samples were then diluted in PBS to 400 μM aliquots. These aliquots were further diluted, in PBS, as needed to working concentrations for their respective assays. Aliquots were stored in fluorescence protection bags at -20 °C until needed. Aliquots were tested before freezing and after being frozen to ensure effectiveness; no difference in effectiveness was observed.

2.6 Procedure

2.6.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were diluted to a 24 μM concentration and then analyzed according to the method published in Gupta-Elera et al. (2012) with the following modifications: 20 μL of AAPH was used instead of 25 μL AAPH, and assays were run for 120 minutes instead of 90 minutes. The final concentration of all samples tested was 2 μM. The ORAC assay was used because it is a well-established method of measuring antioxidant activity *in vitro* (Andrew R. Garrett et al., 2010). The sample to be tested was mixed with a fluorescent molecule (Fluorescein) and an oxygen radical generator (AAPH). The level of fluorescence was measured every 2 minutes over a 120 minute period to create fluorescence decay curves. Experimental samples were run in the same plate as a standard (Trolox) and control samples (Fluorescein alone and Fluorescein plus AAPH). Samples were run in a BMG FLUOstar Optima plate reader with 485 nm emission and 590 nm excitation filters. Samples were run in six different plates with twelve replicates per plate for each compound.

2.6.2 Standard Curve

The area under the curve (AUC) was measured for all experimental and standard samples from their respective fluorescence decay curves. The AUC for each sample and standard was calculated by subtracting the AUC of Fluorescein plus AAPH from the AUC of the sample/standard. The AUC for the standard samples were then plotted to create a standard curve. The AUC of each sample was compared to the standard curve to determine its antioxidant activity in Trolox Equivalents per mg sample (TE/mg).

2.6.3 Cellular Uptake of Antioxidant following Oxygen Radical Generator Exposure

Cellular uptake of antioxidant by Raji cells after oxygen radical generator exposure was measured by following the method published in Gupta-Elera et al. (2011) with some modifications. In short, Raji cells were incubated with AAPH for 10 minutes to simulate oxidative stress. Following incubation, they were washed and then treated with 10 μM antioxidant for 10, 20, 45, or 60 minutes. Following treatment, the samples were washed 2 times and sonicated to lyse. Following sonication, the samples were centrifuged for 30 minutes at 3000 g. The supernatant was then removed as the lysate fraction. The pellet was washed and used as the membrane fraction. Three different plates per compound were run for both membrane and lysate fractions with twenty-four replicates per plate.

2.6.4 Protection Against DNA Damage

Raji cells were incubated for 90 minutes at a 10 μM concentration of the compounds. Following incubation, cells were washed twice and then exposed to 10 μM H₂O₂ for 10 minutes. The cells were then washed and re-suspended in warm RPMI 1640 and prepared for comet assay as described below.

2.6.5 DNA Repair

Raji cells were incubated in 500 μL of 10 μM H₂O₂ for 10 minutes to induce DNA damage. Following incubation, 1 mL of cold RPMI 1640 media was added to the cells. The cells were then centrifuged at 450 g for 5 minutes at 4 °C, washed with cold PBS, and re-suspended in fresh warm media. Cells were then treated with the compounds and allowed to repair for a predetermined amount of time (0, 5, 15, 30, 60, and 90 minutes). Samples were then prepared for comet assay as described below.

2.6.6 Comet Assay

Following both protection and repair protocols, samples were prepared for comet analysis by following the methods described by Xiao et al. (2014) with slight modifications. Briefly, samples were mixed with low melting point agarose and layered on double frosted microscope slides (Xiao et al., 2014). The slides were placed in alkaline lysis buffer for 1 hour, rinsed with ddH₂O and then placed in alkaline electrophoresis buffer for 20 minutes. They were then electrophoresed for 30 minutes. Following electrophoresis, slides were allowed to rest in ddH₂O for 15 minutes, then fixed in -20°C 100% ethanol for 5 minutes and allowed to dry prior to being stained with Propidium Iodide and imaged. All comets were scored using TriTek CometScore Freeware v1.5. Samples were run in three different trials with fifty comets imaged per sample, totaling 150 comets per sample.

Comet assay results are reported in terms of Tail Moment. Tail moment is defined as the product of the tail length and the % of DNA in the tail. These values are given as part of the output by the CometScore software and are widely reported for Comet analysis (Olive et al., 2012).

2.7 Statistics

All statistics were performed using JMP Pro 11. ANOVA and the Tukey-Kramer HSD tests were used to compare compounds and PBS for all ORAC and cellular uptake data. Welch's T test was used to compare treatment and non-treatment groups for the Comet Repair data. A Dunnett's Procedure was used to compare all treatment groups with the PBS treatment for the DNA protection data. All statistics were calculated at the family-wise $\alpha = 0.05$ level. All error bars represent the 95% confidence interval of the mean.

3. Results

3.1 ORAC Antioxidant Values

The raw antioxidant values of pterostilbene, piceatannol, and resveratrol as measured by ORAC are given in Figure 2. Resveratrol exhibited the highest antioxidant activity with a mean value 311.403 TE/mg (CI 302.63 \leq μ \leq 320.18, N=72) followed by piceatannol and pterostilbene with a mean of 283.520 TE/mg (CI 274.75 \leq μ \leq 292.29, N=72) and 126.875 TE/mg (CI 118.10 \leq μ \leq 135.65, N=72) respectively. There was a significant difference ($p < 0.0001$) in the mean TE/mg values among the tested compounds.

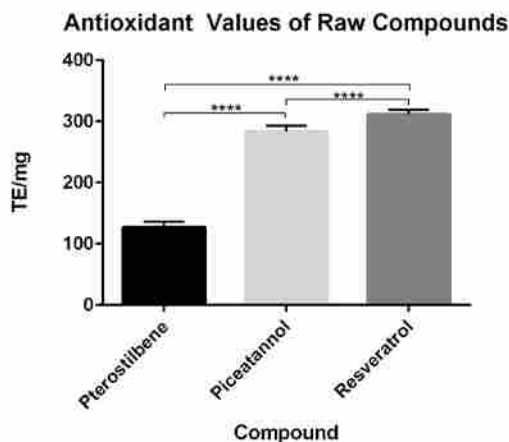


Figure 2. Antioxidant values of raw compounds as measured by ORAC assay reported as mean Trolox Equivalents per milligram (TE/mg). Each mean was significantly different than those of the other two compounds ($p < 0.0001$, $n = 72$ respectively).

3.2 Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure

3.2.1 Lysate Fraction

Figure 3 shows the results from our cellular uptake model for the lysate fraction of Raji cells. There was a significant difference in the mean antioxidant capacity between the lysates of cells treated with the different compounds.

Treatment of Raji cells with pterostilbene had the highest antioxidant activity with a mean of 22,407 TE/L/10⁶ cells followed by piceatannol and resveratrol with mean values of 16,319 and 12,424 TE/L/10⁶ cells, respectively.

Treatment of Raji cells with pterostilbene produced significantly higher antioxidant values ($p < 0.0001$), compared to piceatannol, resveratrol and the control. We also found significantly higher antioxidant values in Raji cells treated with piceatannol compared to those treated with resveratrol ($p = 0.0283$).

3.2.2 Membrane Fraction

Contrary to the lysate, the mean antioxidant values for the membranes of cells treated with any of the three compounds were less than raw Trolox (standard), (Figure 4). The treatment with the highest antioxidant value was pterostilbene, (mean = -4614 TE/L/10⁶ cells). This treatment was the only treatment that was significantly different from the other groups ($p < 0.0001$). Piceatannol, resveratrol, and PBS had mean antioxidant values of -11024, -11390, and -10612 TE/L/10⁶ cells, respectively. Antioxidant values are reported as negative because they are being compared to a standard curve of raw Trolox, as opposed to cells treated with Trolox. Thus, the amount of antioxidant in the membrane fraction is not as high as raw Trolox. There is no precedent for a Trolox standard curve from cells incubated with Trolox so results were compared to a raw Trolox standard curve.

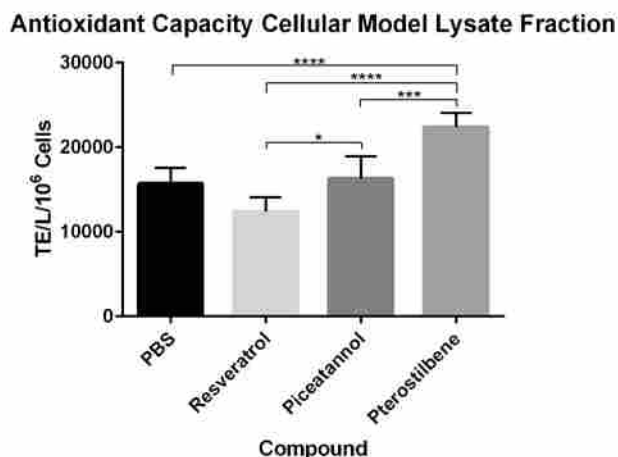


Figure 3. Mean antioxidant values of Raji cell lysates. Antioxidant values of cell lysate following challenge with AAPH and recovery with the specified antioxidant treatments. $n = 72$ for each treatment

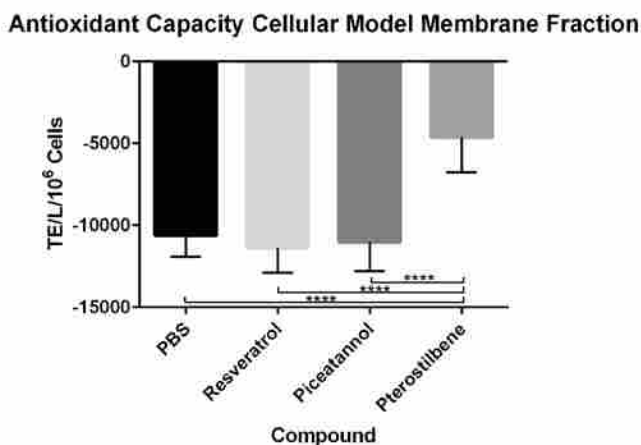


Figure 4. Mean antioxidant values of Raji cell membrane fractions following challenge with AAPH and recovery with the specified antioxidant treatments. $n = 72$ for each treatment

3.3 DNA Protection

The DNA protection assay was performed by pretreating Raji cells with one of the antioxidant compounds or the control (PBS) prior to treatment with hydrogen peroxide (Figure 5). Surprisingly, samples pretreated with pterostilbene and resveratrol exhibited larger tail moments than samples pretreated with PBS alone, ($p < 0.0001$). Samples pretreated with piceatannol had smaller tail moments than samples pretreated with PBS ($p < 0.0001$). No difference was observed between samples pretreated with PBS and samples receiving no pretreatment.

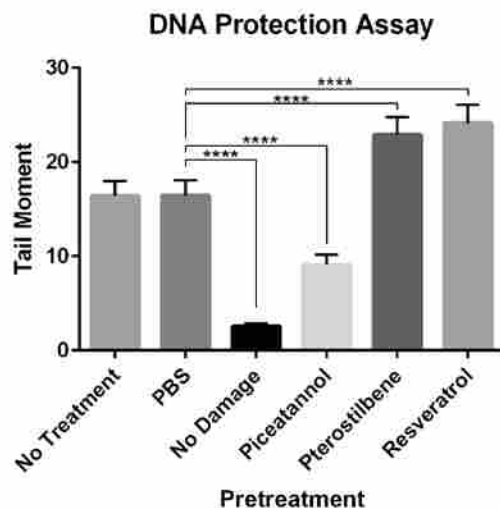


Figure 5. DNA protection. Raji cells were pretreated with antioxidant compounds prior to treating with H_2O_2 . Larger tail moments indicate more DNA damage, $n = 150$ for each treatment

3.4 Comet Repair Assay

The results from our Comet repair assay showed a decrease in the tail moment of cells over time (Figures 6,7, and 8); however, there was no difference in tail moment for samples treated with or without pterostilbene, piceatannol, or resveratrol after 90 minutes of repair ($p = 0.9488, 0.7750, \text{ and } 0.6785$ respectively).

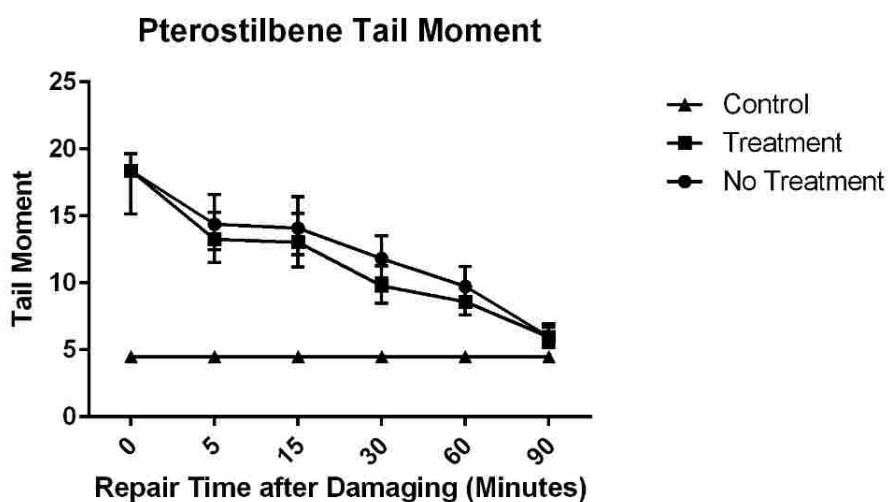


Figure 6. Tail moments for pterostilbene. Tail moments were measured for samples treated either with or without pterostilbene over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.9488$). $n = 150$ for each treatment at each time point

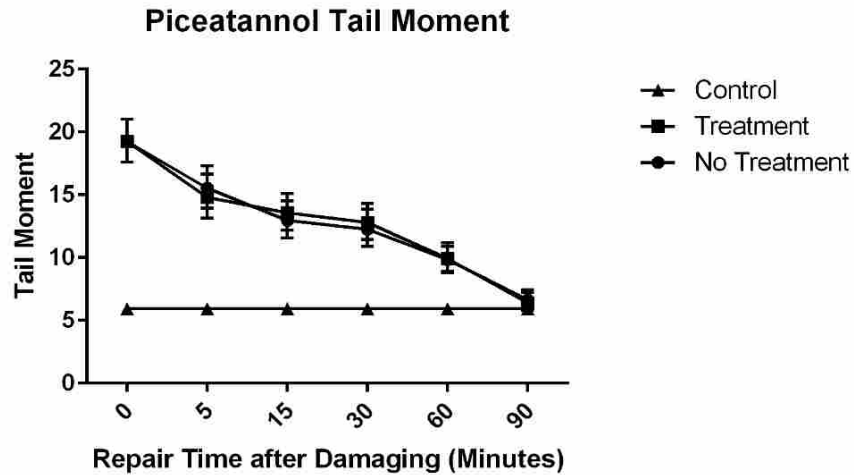


Figure 7. Tail moments for piceatannol. Tail moments were measured for samples treated either with or without piceatannol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.7750$). $n = 150$ for each treatment at each time point

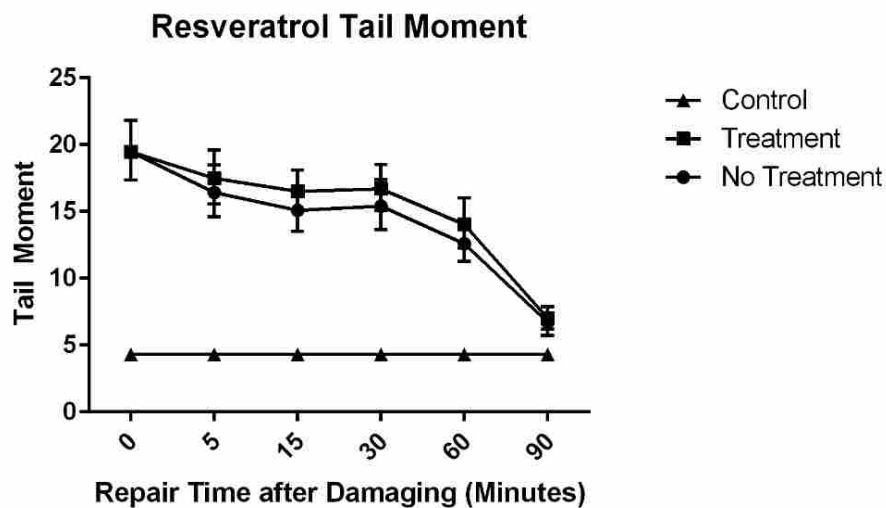


Figure 8. Tail moments for resveratrol. Tail moments were measured for samples treated either with or without resveratrol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.0612$). $n = 150$ for each treatment at each time point

4. Discussion

The results of this study provide interesting information regarding resveratrol and its analogs: piceatannol and pterostilbene.

4.1 Analysis of Antioxidant Abilities

Even though resveratrol demonstrated significantly higher raw antioxidant activity than either piceatannol or pterostilbene, its ability to be absorbed by Raji cells for future antioxidant protection was weak. As suspected, pterostilbene did show a significantly increased ability to be absorbed compared to piceatannol, resveratrol, and control. Piceatannol was also shown to have more absorption ability than resveratrol in Raji cells. We tested this absorptive capacity at different time points, 10, 20, 45, and 60 minutes, as well as at 1 μM and 10 μM , concentrations. ANOVA revealed no significant differences between the antioxidant capacity between the two concentrations or the four time points for each individual compound, so the data from all time points and concentrations was combined into

1 group for each compound. These compound groups were then compared to each other for the results reported here. This evidence helps to support our hypothesis that the structures of pterostilbene and piceatannol affect their ability to protect cells from internal oxidizing damage. These results suggest a need for further studies into the biological roles of both pterostilbene and piceatannol.

4.2 DNA Protection

We investigated the ability of these compounds to protect against DNA damage induced by treatment with hydrogen peroxide. We first wanted to ensure that these compounds were not exerting any cytotoxic effects on the Raji cells, so cells were exposed to each of these compounds. We did not see any loss of viability compared to cells incubated with PBS, as measured by Trypan blue exclusion after 24, 48, or 72 hours, indicating that the results we obtained were not due to loss of cell viability (data not shown). We also wanted to ensure that no DNA damage was induced by these compounds so we performed comet analyses on Raji cells treated with each compound. We observed no difference in tail moments of Raji cells treated with these compounds when compared to cells treated with PBS, indicating that these compounds themselves had not caused DNA damage directly to the cells (data not shown).

To assess the ability of these compounds to protect Raji cells from DNA damage, we pretreated Raji cells with each compound and then exposed them to hydrogen peroxide. While piceatannol pretreatment demonstrated the ability to protect from DNA damage ($p < 0.0001$), resveratrol and pterostilbene pretreatment resulted in increased DNA damage following hydrogen peroxide exposure, ($p < 0.0001$). Reasons for this are unclear at this point. Increase in tail moment after pretreatment with resveratrol and pterostilbene may be due to anticancer effects or cell death inducing effects of these agents. We did not observe any loss of viability or increase in DNA damage after treatment with these compounds alone, so pretreatment may predispose Raji cells to undergo cell death following oxidative stress. Xia et al. (2010) reported that resveratrol has anticancer effects for prostate, breast and epithelial cancers. These results suggest that this effect may extend to lymphocytic cancers as well. Further work will help to elucidate and characterize these mechanisms.

4.3 DNA Repair Analysis

In an effort to investigate whether or not these compounds could help to repair previously damaged DNA, we tested both a 1 μM (data not shown) and a 10 μM concentration. No positive effects on DNA repair were observed at either concentration. We also looked at several time points to see if there were differences over time. We saw no significant effect on DNA repair rates of Raji cells that received antioxidant treatment versus those that did not. This result was observed for all time points and concentrations tested.

These results indicate that among the many biologically relevant roles of resveratrol, piceatannol, and pterostilbene, DNA repair modulation does not appear to be one of them. This study provided no additional evidence of resveratrol's usefulness as an intracellular antioxidant, but has shown the potential usefulness of pterostilbene in protecting cells from intracellular oxidizing damage. This work also provided information about the possible anticancer activities of resveratrol and pterostilbene. This preliminary study demonstrates the need to further investigate the biologically active roles of all three compounds. Such work may prove to establish mechanisms which may increase longevity and reduce aging. Foods containing antioxidants should be studied further to provide greater insight into the role of a healthy balanced diet in improving health. Such studies may be valuable in helping to alleviate many lifestyle diseases worldwide.

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APPENDIX B: Manuscript Submitted to *The Bryologist*

Examination of the Antioxidant capacity of acetone extracts of North American lichens using the

Oxygen Radical Absorbance Capacity (ORAC) assay

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Abstract

Oxidative stress leads to elevated production of free radicals, which are associated with a variety of human health issues, including cancer and Alzheimer's. Natural products rich in phenolic compounds neutralize potentially harmful free radicals, suggesting that the use of such compounds may be therapeutically effective as a source of exogenous antioxidant defenses. Lichens produce a unique group of phenolic compounds. The goal of this study was to investigate the antioxidant activity of acetone extracts of twelve lichen species collected from various parts of the United States. Antioxidant activity was determined using the Oxygen Radical Absorbance Capacity (ORAC) assay. The results of this study showed that all lichen extracts, except the *Thamnolia vermicularis* extract, demonstrated strong antioxidant activity and inhibited the oxidative degradation of the fluorescent molecule (fluorescein-sodium salt) by the oxygen free radical initiator AAPH (2,2'-azobis(2-aminidopropane) dihydrochloride). The *Parmotrema reticulatum* extract showed the strongest antioxidant activity. As suggested by many studies antioxidants are effective in preventing various chronic diseases, the potential antioxidant role of lichen phenolic compounds certainly merits further consideration.

Keywords: Lichens, antioxidant, phenolics, ORAC

Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\cdot), and the superoxide anion (O_2^\cdot) cause oxidative stress. Oxidation reactions, although crucial to life, can sometimes have damaging effects on cells often leading to the development of various chronic diseases like arthritis, cancer, and immunodeficiency syndromes (Kosanić, Ranković, & Vukojević, 2011). Hence a balance between oxidative stress and antioxidants is necessary in living systems. To effectively deal with the damaging effects of ROS, living organisms maintain a complex system of antioxidants. Research has shown that antioxidants, obtained either from natural or synthetic sources, are effective in reducing oxidative damage from ROS in the human body (Karihtala and Soini, 2007). However, there are reports showing that synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are toxic and can have carcinogenic effects (Zhang, Li, Han, & Zhang, 2009). For these reasons, recent studies have focused on the screening of natural products for their antioxidant properties (Naveena, Sen, Kingsly, Singh, & Kondaiah, 2008). According to (Saha et al., 2008), some phenolic compounds have demonstrated antioxidant properties in biological systems. Lichens produce an impressive variety of polyphenolic compounds (G. Shrestha & St. Clair, 2014) therefore, extending our search for new potentially viable, natural antioxidants to lichens seems prudent.

Lichens produce an impressive variety of secondary metabolites, most of which are unique to lichens. More than 1000 secondary metabolites have been isolated from lichens and their cultured symbionts (Molnar & Farkas, 2010), with only minimal overlap with secondary compounds from higher plants (Elix, 1996). Lichens have been used for various medicinal purposes since ancient times (Huneck, 1999; Vartia, 1973). For example, *Lobaria pulmonaria*, *Parmelia sulcata*, and *Letharia vulpina* have been used to treat pulmonary, cranial, and stomach

disorders respectively (Huneck, 1999; Kirmizigul, Koz, Anil, Icli, & Zeybek, 2003; Malhotra, Subban, & Singh, 2008). More recently research has shown that lichen metabolites demonstrate significant biological activity, including antiviral, antibiotic, antitumor, antioxidant, anti-herbivory, anti-HIV etc. (Gajendra Shrestha & St. Clair, 2013). Of their various biological properties, their antibiotic and antioxidant potentials have been most commonly studied.

North America is home to an impressive variety of lichen species. Many studies have been published examining the taxonomical (Barton et al., 2014; Hutten et al., 2013), phylogenetic (Leavitt, Lumbsch, & St. Clair, 2013), and ecological (P. R. Nelson, Roland, Macander, & McCune, 2013) aspects of lichens in North America; however, the biological roles of lichens have been poorly studied. Studies from different parts of the world (Bhattaria et al., 2013; Ghate et al., 2013; Jayaprakasha & Rao, 2002; Kumar et al., 2014; Raj et al., 2014) have shown that crude extract of lichens as well as their metabolites possess antioxidant properties. Therefore, we hypothesized that crude lichen extracts from North American lichens will have strong antioxidant comparable to Trolox, a water-soluble analogue of vitamin E used as a standard for antioxidant experiments.

This is the first study to document the antioxidant properties of acetone extracts of twelve lichen species collected from various parts of the United States using the Oxygen Radical Absorbance Capacity (ORAC) assay.

Materials and Methods

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was obtained from Enzo Life Sciences, Inc. (Plymouth, PA). The 2,2'-azobis(2-aminidopropane) dihydrochloride (AAPH) was

purchased from Wako Chemicals USA, Inc. (Richmond, VA), and the Fluorescein-sodium salt, was purchased from Sigma-Aldrich, Inc. (Milwaukee, WI).

Lichen materials

Samples of twelve species of lichens (Table 1) were collected from various parts of the United States. All species identifications were confirmed by Larry L. St. Clair, curator of the Herbarium of Nonvascular Cryptogams at Brigham Young University, Provo, Utah and Roger Rosentreter, Boise State University, Boise, Idaho. Voucher collections of each of the 12 species have been curated and deposited in the Herbarium of Non-vascular Cryptogams at Brigham Young University, Provo, Utah, USA.

Extraction of lichen compounds

Prior to extraction, lichen thalli were washed and dried. Extraction of lichen compounds followed the protocol from Shrestha et al. (2014). Briefly, Five grams of dried thalli from each lichen species were powdered and then extracted with 50 ml of acetone. Once the extraction process was completed, in order to prevent further oxidation of the extracts, evaporation of the acetone was facilitated by bubbling a stream of nitrogen through each sample. Extracts were then dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 16 mg/ml. Residual extract material was then stored at -20°C.

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay was performed following the protocol of (Andrew R Garrett, Murray, Robison, & O'Neill, 2010). Five different concentrations of each lichen extract (50, 25, 12.5, 6.25, and 3.125 µg/ml) were prepared from the stock solution (16 mg/ml). Twenty µL of each concentration was then combined with 200 µl of a 0.1061 µM fluorescein solution. A BMG Fluostar Optima plate reader was used to measure fluorescence at 37 °C over three two- minute cycles. Initial fluorescence was determined using 485 nm excitation and 590 nm emission filters.

After the first three cycles, 20 μL of 26.55mM AAPH was added to the test solutions. The final concentration of each lichen extract ranged from 4.2 to 0.26 $\mu\text{g/ml}$ after dilution with fluorescein and AAPH. Fluorescence was then measured for an additional 57 cycles. All fluorescent measurements were expressed relative to the initial gain adjustment reading. Control samples contained only fluorescein and fluorescein with AAPH. Trolox is a water-soluble analogue of vitamin E which is used as a standard for antioxidant experiments. Five different concentrations of trolox standards - 25 $\mu\text{g/ml}$, 12.5 $\mu\text{g/ml}$, 6.25 $\mu\text{g/ml}$, 3.12 $\mu\text{g/ml}$, and 1.56 $\mu\text{g/ml}$ were used in this study. Fluorescence curves were generated for all extracts and standards and the area under the curve was calculated to determine the Trolox Equivalents per microgram (TE/ μg) of each lichen extract. The area under the curve (AUC) was calculated by determining the difference between the area under the fluorescein decay curves and the area under the sample curve and the fluorescein + AAPH curve. The AUC value indicates the total antioxidant activity of the sample minus the area under the fluorescein + AAPH antioxidant curve. Sample fluorescence decay curves of trolox and lichen extracts are shown in Fig 1. Each value is the mean of at least 3 experimental runs each conducted in triplicate.

Results and Discussion

This study reports on the antioxidant capacity of acetone extracts of twelve lichen species collected from different parts of the United States (Table 1) using the Oxygen Radical Absorbance Capacity (ORAC) assay. The ORAC assay is a commonly used tool to measure the antioxidant activity of vitamins, phytochemicals, and other organic and inorganic compounds (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). This assay measures the oxidative degradation of a fluorescent molecule (usually fluorescein-sodium salt or beta-phycoerythrin) by an oxygen radical initiator such as AAPH (Huang, Ou, & Prior, 2005). With the addition of the oxygen radical initiator, the intensity of the fluorescent molecules begins to decrease; however,

by adding an antioxidant compound the rate at which the fluorescent molecule degrades can be significantly reduced. Thus, the longer a fluorescent molecule maintains its intensity, the more effective the antioxidant compound (Andrew R Garrett et al., 2010). One important aspect of the ORAC assay is calculating Trolox Equivalents (TE). Trolox, a water-soluble vitamin E analogue, is a standard used in antioxidant experiments and its capacity for preventing oxidation-related damage has been well documented (Hamad, Arda, Pekmez, Karaer, & Temizkan, 2010). In this study we used Trolox as our standard for evaluating and comparing the antioxidant capacity of lichen extracts.

Our results showed that except for *T. vermicularis*, all other lichen extracts demonstrated antioxidant properties (Table 2). We also show that out of the twelve lichen extracts, six species namely, *P. reticulatum*, *M. richardsonii*, *U. mammulata*, *R. peltata*, *E. catawbiense*, and *L. pulmonaria* reduced the oxidative degradation of the fluorescent molecule by AAPH even at the lowest extract concentration (0.26 µg/ml). Several reports have already documented the antioxidant properties of various lichen extracts. Specifically, (Ranković, Kosanić, & Stanojković, 2011) studied the antioxidant properties of acetone extracts of three lichens – *Cladonia furcata*, *Lecanora atra*, and *Lecanora muralis*. The results showed that all of the extracts demonstrated antioxidant activity with *Lecanora atra* having the highest free radical scavenging capacity. (Buçukoglu, Albayrak, Halici, & Tay, 2013) researched the antioxidant activity of methanolic extracts and various purified lichen metabolites obtained from six *Umbilicaria* species. All extracts demonstrated moderate DPPH radical-scavenging activity. They also showed that umbilicic acid had the highest antioxidant activity. (Kumar et al., 2014) evaluated the antioxidant properties of n-hexane, methanol, and water extracts of fourteen saxicolous (rock) lichens from the trans-Himalayan Ladakh region and documented that the species tested showed a broad spectrum of free

radical scavenging and high antioxidant capacity. Most studies evaluating the antioxidant properties of lichen compounds have demonstrated a strong correlation between antioxidant activity and phenolic content (Brisdelli et al., 2013; Buçukoglu et al., 2013; Ghate et al., 2013; Kosanić et al., 2011). Documentation of the major secondary chemicals in the 12 species tested in our study has been previously published (Gajendra Shrestha, Raphael, Leavitt, & St. Clair, 2014); and all 12 species contained various phenolic compounds (Table 3). Phenolic compounds demonstrate strong antioxidant qualities because of their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Saha et al., 2008). Phenolic compounds transfer hydrogen to free radicals to break the chain reaction of lipid oxidation at the first initiation step (Ranković et al., 2011). Hence our results provide strong evidence that the acetone extracts from the lichen species included in this study have promising antioxidant properties.

We also ranked our 12 lichen species on the basis of TE/ μg at 4.2 $\mu\text{g}/\text{ml}$ concentration of the lichen extracts (Fig. 2). The three species with the highest antioxidant capacity were *P. reticulatum*, *M. richardsonii*, and *U. mammulata* with TE/ μg , values of 993.4 ± 54.5 , 829.1 ± 99.4 , and 794.6 ± 79.2 respectively. These values can be interpreted as 4.2 μg of the *P. reticulatum* extract would produce the same antioxidant activity as 993.4 μg of Trolox. Hence, compared to Trolox, lichen extracts demonstrate stronger antioxidant properties. Similar results have also been reported by Amo de Paz et al. (2010). These three lichen species contained atranorin, alectoronic acid, and gyrophoric acid, respectively, as major secondary metabolites (Table 3). Many lichen species containing atranorin, alectoronic acid, and gyrophoric acid have already been screened for their antioxidant properties and have shown positive results (Buçukoglu et al., 2013; Kosanić et al., 2011; Manojlovic, Vasiljevic, Maskovic, Juskovic, & Bogdanovic-Dusanovic, 2012; Melo et al.,

2011; Ravaglia et al., 2014). The phenolic groups typically associated with lichen metabolites are considered to be key elements in determining their antioxidative capacity (Marković & Manojlović, 2010). The above mentioned lichen acids have two or more phenolic groups which likely explains their antioxidant activity.

In our study the *T. vermicularis* extract, at the tested concentrations, did not show antioxidant capacity. However (Luo et al., 2006) reported antioxidant activity in methanol extracts of the same species. These conflicting results might be due to differences in the chemical extraction process and different assay method used for measuring antioxidant activity.

Based on the current literature, of the twelve species examined in this study six species, *A. sarmentosa*, *E. catawbiense*, *M. richardsonii*, *R. sinensis*, *R. peltata*, and *X. wyomingica* have not been previously surveyed for their antioxidant properties.

In conclusion, of the 12 lichen species evaluated in this study 11 showed strong antioxidant properties compared to Trolox; the only exception was *T. vermicularis*. Thousands of lichen species have been reported for North America; however, this study represents the first group of lichen species which have been evaluated for their antioxidant properties. Based on the results of our research, lichen extracts from North America merit more detailed consideration as a potential source of antioxidant compounds. Future studies should include not only the Trolox equivalent values from ORAC but also determination of polyphenolic content as well as free radical and superoxide scavenging activity.

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3887-3892

Table 1: List of lichen species used in this study with collection site information

Lichens	Collection Site	Collection Date
<i>Alectoria sarmentosa</i> (Ach.) Ach.	Montana, Mission Mountain Wilderness Area, Cold lake trailhead, 47° 33' 39.6" N, 113° 51' 19.5" W	Aug 19, 2010
<i>Cladonia furcata</i> (Hudson) Schrader	Tennessee, Carter County., Near the vicinity of Roan Mountain State Park visitor center, 36° 10' 15.3" N, 82° 5' 57.78" W	Jun 20, 2011
<i>Everniastrum catawbiense</i> (Degel.) Hale ex Sipman	Tennessee, Carter County, 9 miles south from Roan Mountain State Park (TN 143S/Roan Road), Near Tennessee and North Carolina boarder Along Appalachian trail, 36° 6' 24.84" N, 82° 6' 32.46" W	Jun 22, 2011
<i>Letharia columbiana</i> (Nutt.) J. W. Thomson	Idaho, Blaine County, Sawtooth National Recreational Area, Vicinity of Alpine creek trailhead, 43° 53' 53.04"N, 114° 54'25.62" W	Jun 30, 2010
<i>Lobaria pulmonaria</i> (L.) Hoffm.	Idaho, Idaho County, Clearwater National Forest, Along USFS RD #362, South of fish hatchery, 46° 30' 23.46" N, 114° 40' 52.08" W	Aug 17, 2010
<i>Masonhalea richardsonii</i> (Hooker) Kärnefelt	Alaska, Fairbanks North Star County, Alpine tundra at Eagle summit, north-west of	Jul 31, 2011

	parking area, Steese Highway, 65° 29' 12.12" N, 145° 24' 55.08" W	
<i>Parmotrema reticulatum</i> (Taylor) M. Choisy	Montana, Mission Mountain Wilderness Area, Crystal lake trailhead, 47° 20' 1.38" N, 112° 43' 48.24"	Aug 19, 2010
<i>Ramalina sinensis</i> Jatta	Utah, Utah Co., Uinta NF., Along filth water hot-spring, about 0.3 miles from trailhead, 40° 3' 55.488" N, 111° 21' 8.316" W	Sep 23, 2010
<i>Rhizoplaca peltata</i> (Ramond) Leuckert & Poelt	Wyoming, Sweetwater County, Ashley National Forest, Fleming Gorge National Recreational Area, Along USFS Road # 146 (Lucerne valley Recreational Road), 41° 0' 14.616" N, 109° 32' 11.2914" W	Aug 10, 2010
<i>Umbilicaria mammulata</i> (Ach.) Tuck.	Tennessee, Carter County, Roan Mountain State Park, Raven Rock Trail, 36° 9' 57.54" N, 82° 5' 30.54" W	Jun 21, 2011
<i>Thamnolia vermicularis</i> (Sw.) Ach. ex Schaerer	Montana, Granite Co., Anaconda Pintler wilderness area, Goat flat, 46° 3' 15.72" N, 113° 16' 38.22" W	Aug 18, 2010
<i>Xanthoparmelia wyomingica</i> (Gyelnik) Hale	Montana, Granite Co., Anaconda Pintler wilderness area, Goat flat, 46° 3' 15.72" N, 113° 16' 38.22" W	Aug 18, 2010

Table 2: TE/ μg values at different concentrations ($\mu\text{g}/\text{ml}$) of lichen extract

Species	Antioxidant Value (TE/ μg)				
	4.2 $\mu\text{g}/\text{ml}$	2.1 $\mu\text{g}/\text{ml}$	1.05 $\mu\text{g}/\text{ml}$	0.525 $\mu\text{g}/\text{ml}$	0.26 $\mu\text{g}/\text{ml}$
<i>P. reticulatum</i>	993.4 \pm 54.5	536.3 \pm 37.5	392.7 \pm 11.9	278.4 \pm 41.7	130.8 \pm 18.1
<i>M. richardsonii</i>	829.1 \pm 99.4	462.6 \pm 89	243.8 \pm 35.2	110 \pm 24.2	23.5 \pm 12.5
<i>U. mammulata</i>	794.6 \pm 79.2	561.2 \pm 12.6	326.2 \pm 43.2	179.8 \pm 24.1	116.2 \pm 46.2
<i>R. peltata</i>	740.8 \pm 54.4	400.7 \pm 70.8	242.1 \pm 30.6	115.7 \pm 14.8	98.7 \pm 13.4
<i>E. catawbiense</i>	681.5 \pm 25.9	438.8 \pm 51.3	253.8 \pm 21.8	181.6 \pm 10.9	68.2 \pm 23.3
<i>L. pulmonaria</i>	529.4 \pm 69.5	333.7 \pm 68.9	114 \pm 25.7	67.4 \pm 25.3	12.4 \pm 6.9
<i>A. sarmentosa</i>	279.9 \pm 39.1	158.8 \pm 21.1	71.1 \pm 12.7	77.8 \pm 27.1	-
<i>X. wyomingica</i>	194.5 \pm 15.8	121.4 \pm 19.9	65.8 \pm 20.6	39.5 \pm 7.4	-
<i>C. furcata</i>	160.5 \pm 17.6	99.4 \pm 13	53.9 \pm 18.2	-	-
<i>L. columbiana</i>	132.9 \pm 18.0	-	-	-	-
<i>R. sinensis</i>	109.6 \pm 11.6	58.07 \pm 6.26	28.5 \pm 17	-	-
<i>T. vermicularis</i>	-	-	-	-	-

Table 3. Primary lichen compounds identified for each species using Thin Layer

Chromatography

Lichens	Compounds
<i>Alectoria sarmentosa</i>	Usnic acid, Barbatic acid, Squammatic acid, Thamnolic acid
<i>Cladonia furcata</i>	Fumarprotocetraric acid, Protocetraric acid
<i>Everniastrum catawbiense</i>	Atranorin, Gyrophoric acid
<i>Letharia columbiana</i>	Vulpinic acid
<i>Lobaria pulmonaria</i>	Norstictic acid, Stictic acid, Constictic acid, Cryptostictic acid, Confumarprotocetraric acid
<i>Masonhalea richardsonii</i>	Alectoronic acid
<i>Parmotrema reticulatum</i>	Atranorin
<i>Ramalina sinensis</i>	Usnic acid
<i>Rhizoplaca peltata</i>	Usnic acid, Atranorin, Pannarin
<i>Thamnolia vermicularis</i>	Thamnolic acid, Baeomycesic acid
<i>Umbilicaria mammulata</i>	Gyrophoric acid
<i>Xanthoparmelia wyomingica</i>	Usnic acid, Salazinic acid, Consalazinic acid, Norstictic acid

Fig. 1: Sample fluorescence decay curves for controls and different concentrations of the *Parmotrema reticulatum* extract.

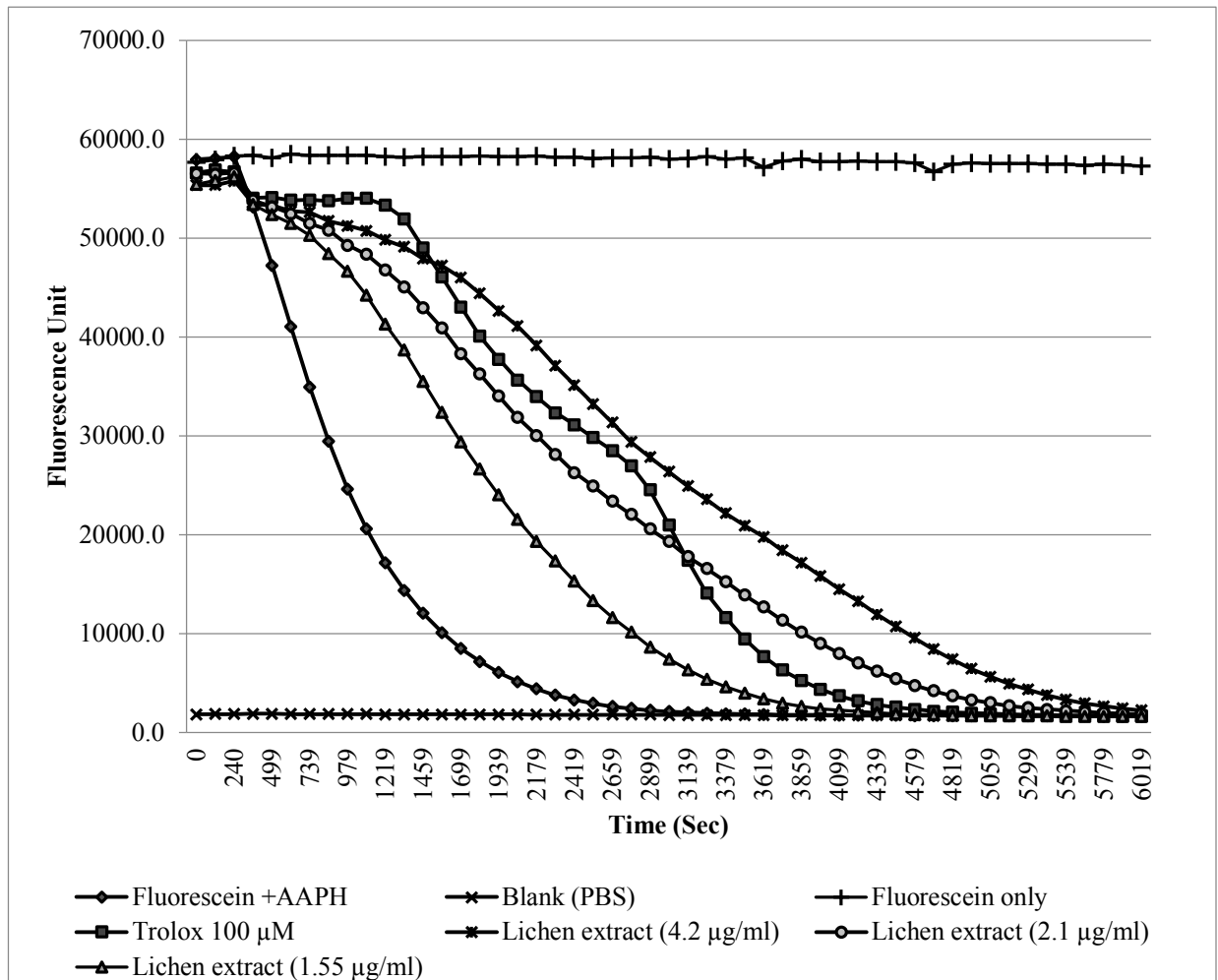
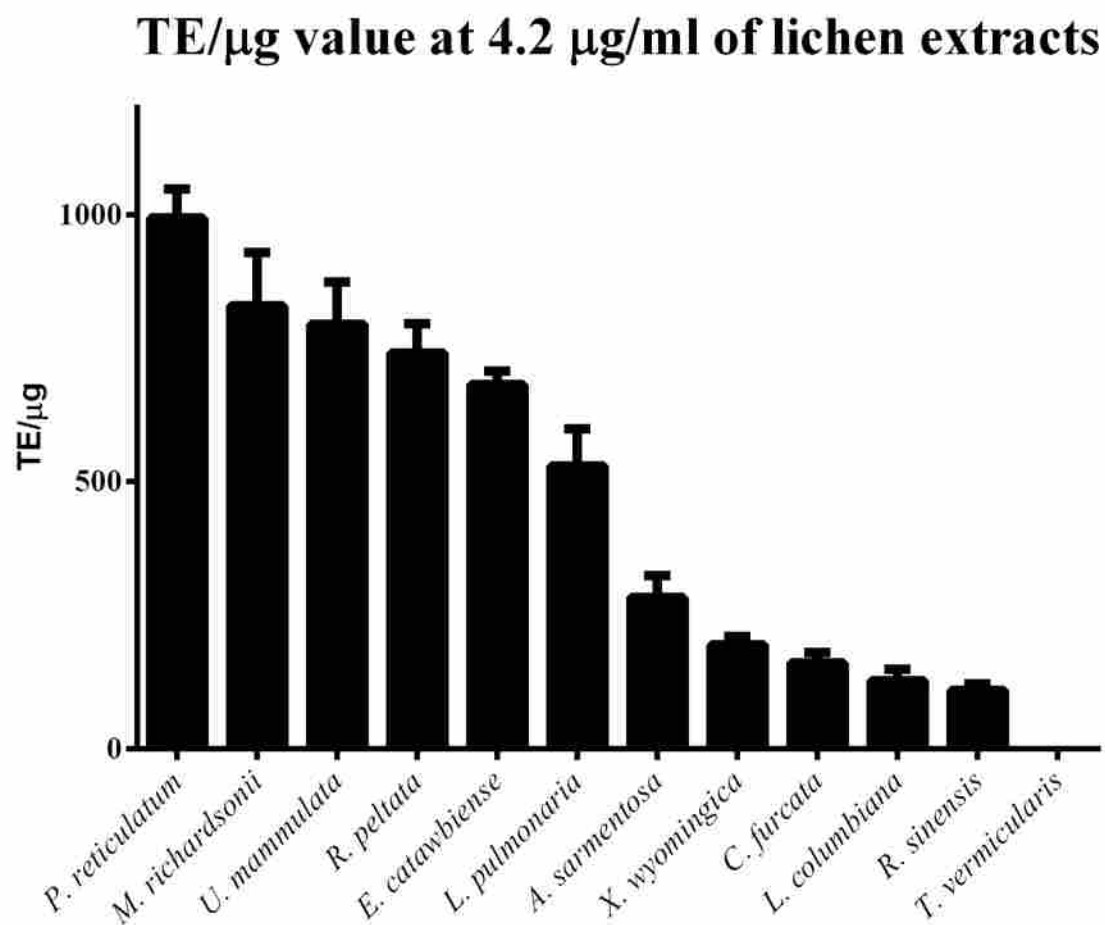


Fig. 2: Comparison of antioxidant capacity (TE/ μ g) of extracts from twelve lichen species.

Values are reported as mean values \pm SD



APPENDIX C: Presentations

Poster Presentation at the 10th annual Undergraduate Student Caucus and Poster Competition of the AACR, entitled, “The Antioxidant Capacity and DNA Repair Effects of Resveratrol and its Analogs: Piceatannol and Pterostilbene”. Peterson, J., Livingston, J., Martinez, G., Peck, C., Thompson, B., Shrestha, G., and O’Neill, K.L. April 18-22 2015, Philadelphia Pennsylvania.

Lecture Series. Taught unit on Vaccination for MMBIO 463 Advanced Immunology Course. Winter Semester 2015.

Oral Presentation at the Donald C. Sloan Speech Showcase entitled, “Spread the Word”. December 2014.

Poster Presentation at the 2014 Intermountain Branch Meeting of the American Society for Microbiology at Brigham Young University. March 8th 2014. “Piceatannol and Pterostilbene: Resveratrol Analogs, Potent Antioxidants” J. Peterson, J. Livingston, B. Graham, L. Camberos, G. Martinez, G. Shrestha, Andres Martinez, E. Weagel, R. Robison, and K. L. O’Neill.

Poster Presentation at the 53rd Midwinter Conference of Immunologists at Asilomar, entitled “Apoptotic and Necrotic Cancer Cells Differentially Affect Macrophage Aggressiveness” Justin Livingston Gajendra Shrestha, Eugene Lee, Evita Weagel, Kim O’Neill. January 25-28, Pacific Grove California.

Poster Presentation at the 53rd Midwinter Conference of Immunologists at Asilomar, entitled “Metastatic Breast Cancer Cells Can Polarize Macrophages to an M2 Phenotype” Gajendra Shrestha, Evita Weagel, Justin Livingston, Larry St. Clair, Richard Robison, Kim O’Neill. January 25-28, Pacific Grove California.

Poster Presentation at Presented at the 16th Annual Meeting of the American Society of Gene and Cell Therapy, entitled “Investigating macrophage induction of metastasis: a precursor to identify therapeutic targets.” Trevor Memmott, Evita Weigel, Justin Livingston, PingGuo Liu, Atif Elnaggar, Richard Robison Kim O'Neill. May 15-18, Salt Lake City Utah.

Poster Presentation at the 8th annual Undergraduate Student Caucus and Poster Competition of the AACR, entitled, “Apoptotic and Necrotic Signals may Modulate Macrophage Polarization”. E. J. Lee, S. Barlow, A.M. El-Naggar, E. Weigel, P.G. Liu, J. Livingston, R. Robison, and K.L O'Neill. April 5-10, Washington D.C.

Oral Presentation at the 2013 Intermountain Branch Meeting of the American Society for Microbiology at Idaho State University, Pocatello, Idaho. March 9th 2013. Macrophages may be polarized by apoptotic and necrotic cancer cells. Livingston, J., Lee E. J., Barlow, S., El-Naggar, A. M., Weigel, E., Liu, P., Robison R., O'Neill, K. L. Presentation won first prize.