Polyphenols are natural antioxidants found in foods such as berries, tea, and wine. Although polyphenols have been studied extensively in chemical systems, less is known about their activity in biological systems. I am especially interested in polymeric polyphenols, which have a high capacity for radical scavenging and may thus be good inhibitors of the inflammatory cascade. To test the hypothesis, a rabbit monocyte cell system was used. A superoxide burst was monitored using cytochrome c reduction or chemiluminescence. TNF-alpha production was assessed with an ELISA. It was found that a typical polymeric polyphenol, pentagalloyl glucose, was both a superoxide scavenger and a stimulator of TNF-alpha production. Studies of other polyphenolics and of polyphenol-protein complexes in the same system will allow development of structure-activity relationships for radical scavenging and cytokine stimulating activities noted for polymeric polyphenols. Identifying polymeric polyphenols that effectively scavenge radicals without stimulating cytokine production is essential to using polyphenols as anti-inflammatory agents.
POLYMERIC POLYPHENOLS AS ANTI-INFLAMMATORY AGENTS

A Thesis

Submitted to the

Faculty of Miami University

in partial fulfillment of

the requirements for the degree of

Master of Science

Department of Chemistry and Biochemistry

By Akeysha A. Perkins

Miami University

Oxford, Ohio

2007

Advisor _______________________
Dr. Ann Hagerman

Reader _________________________
Dr. John Hawes

Reader _________________________
Dr. Michael Crowder

Reader _________________________
Dr. Richard Taylor
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>1</td>
<td>Superoxide Production by Monocytes</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Inflammatory Cascade and Reactive Oxygen Species</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Superoxide Anion</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Hypothesis</td>
<td>2</td>
</tr>
<tr>
<td>1.3</td>
<td>Cytochrome c for Detection of Superoxide</td>
<td>3</td>
</tr>
<tr>
<td>1.4</td>
<td>Luminol for Detection of Superoxide</td>
<td>3</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Luminescence</td>
<td>3</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Luminescence of Luminol</td>
<td>4</td>
</tr>
<tr>
<td>1.5</td>
<td>Methods</td>
<td>5</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Reagents</td>
<td>5</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Luminometer Validation</td>
<td>5</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Enzyme-generate Superoxide</td>
<td>6</td>
</tr>
<tr>
<td>1.6</td>
<td>Results and Discussion</td>
<td>7</td>
</tr>
<tr>
<td>1.7</td>
<td>Chapter 1 References</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Superoxide Scavenging by Polyphenols</td>
<td>22</td>
</tr>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>22</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Reactive Oxygen Species Damage</td>
<td>22</td>
</tr>
<tr>
<td>2.1.2</td>
<td>Antioxidants vs. Anti-Inflammatory Agents</td>
<td>22</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.3</td>
<td>Results and Discussion</td>
<td>47</td>
</tr>
<tr>
<td>3.4</td>
<td>Chapter 3 References</td>
<td>60</td>
</tr>
<tr>
<td>Appendix 1</td>
<td>Free Radical Scavenging and Protein Precipitation Properties of Grape Seed Extract</td>
<td>61</td>
</tr>
<tr>
<td>Appendix 2</td>
<td>Composition of Cottonwood Tannins</td>
<td>79</td>
</tr>
<tr>
<td>A2.1</td>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>A2.2</td>
<td>Methods</td>
<td>79</td>
</tr>
<tr>
<td>A2.3</td>
<td>Results and Discussion</td>
<td>81</td>
</tr>
<tr>
<td>A2.4</td>
<td>Appendix 2 References</td>
<td>86</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td><em>Luminescence of luminol</em></td>
<td>12</td>
</tr>
<tr>
<td>1.2a</td>
<td><em>Oxidation of xanthine using microbial XO</em></td>
<td>13</td>
</tr>
<tr>
<td>1.2b</td>
<td><em>Microbial XO luminescence</em></td>
<td>14</td>
</tr>
<tr>
<td>1.3a</td>
<td><em>Oxidation of xanthine using bovine milk X</em></td>
<td>15</td>
</tr>
<tr>
<td>1.3b</td>
<td><em>Bovine milk XO luminescence</em></td>
<td>16</td>
</tr>
<tr>
<td>1.4</td>
<td><em>Normalized luminescence</em></td>
<td>17</td>
</tr>
<tr>
<td>1.5a</td>
<td><em>Oxidation of xanthine with PGG</em></td>
<td>18</td>
</tr>
<tr>
<td>1.5b</td>
<td><em>Superoxide production with PGG</em></td>
<td>19</td>
</tr>
<tr>
<td>2.1</td>
<td><em>Structure of PGG and procyanidin</em></td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td><em>NBT reduction assay chemistry</em></td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td><em>NBT reduction assay with addition of Quercetin</em></td>
<td>33</td>
</tr>
<tr>
<td>2.4</td>
<td><em>NBT reduction assay with addition of PGG</em></td>
<td>34</td>
</tr>
<tr>
<td>2.5</td>
<td><em>GSE percent scavenging</em></td>
<td>35</td>
</tr>
<tr>
<td>2.6</td>
<td>*GSE IC&lt;sub&gt;50&lt;/sub&gt; values</td>
<td>36</td>
</tr>
<tr>
<td>3.1</td>
<td><em>Erratic replication</em></td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td><em>Combined standard curve</em></td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td><em>TNF-α standard kinetic readings</em></td>
<td>56</td>
</tr>
<tr>
<td>3.4</td>
<td><em>Basal levels of TNF-α production</em></td>
<td>57</td>
</tr>
<tr>
<td>3.5</td>
<td><em>TNF-α production by cells stimulated by LPS in the presence of PGG</em></td>
<td>58</td>
</tr>
<tr>
<td>3.6</td>
<td><em>TNF-α production in cells treated with various amounts of PGG</em></td>
<td>59</td>
</tr>
<tr>
<td>A2.1</td>
<td><em>Thiolysis reaction</em></td>
<td>82</td>
</tr>
<tr>
<td>A2.2</td>
<td><em>HPLC standards</em></td>
<td>83</td>
</tr>
<tr>
<td>A2.3</td>
<td>Chromatogram of representative narrowleaf cottonwood sample after thiolysis reaction</td>
<td>84</td>
</tr>
<tr>
<td>A2.4</td>
<td>Relative moles of condensed tannin extender units and chain length</td>
<td>85</td>
</tr>
</tbody>
</table>
Abbreviations

“Alcohol-Soluble” (AS)
“Pharmaceutical” (PH)
“Standard” (ST)
“Water-Soluble” (WS)
2,2’-azonibis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+)
Acetonitrile (MeCN)
Area under the curve (AUC)
Bovine serum albumin (BSA)
Copper sulfate (CuSO4)
Deoxyribonucleic acid (DNA)
Electron paramagnetic resonance (EPR)
Enzyme linked immunosorbant assay (ELISA)
Epigallocatechin (EGC)
Grape seed extract (GSE)
Hank’s balanced salt solution (HBSS)
High performance liquid chromatography (HPLC)
Horseradish peroxidase (HRP)
Hydroxyl radical (OH)
Inflammatory bowel disease (IBD)
Inhibitory concentration (50%) (IC50)
Interleukin-1 (IL-1)
Lipopolysaccharide (LPS)
Molybdenum (Mo)
Nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
Nitro blue tetrazolium (NBT)
β-1,2,3,4,6-pentagalloyl-O-D-glucopyranose (PGG)
Peripheral mononuclear blood cells (PMBC)
Phenazine methosulfate (PMS)
Phorbol 12-mysristate 13-actetate (PMA)
Phosphate buffered saline(PBS)
Reactive oxygen species (ROS)
Reduced β-nicotinamide adenine dinucleotide (NADH)
Superoxide anion (O$_2^-$)
Tetramethyl benzidine (TMB)
Thiobarbituric acid reacting species (TBARS)
Toluene-α-thiol (TαT)
Trifluoroacetic acid (TFA)
Trizma hydrochloride (Tris-HCl)
Tumor necrosis factor-alpha (TNF-α)
Xanthine oxidase (XO)
1. Superoxide Production by Monocytes

1.1. Introduction

1.1.1. Inflammatory Cascade and Reactive Oxygen Species

The inflammatory cascade contains a series of events which are responsible for the body’s physiological manifestation of redness, pain, and swelling that may derive from irritation, infection, or immune response. Some of the events associated with the inflammatory cascade include migration of phagocytic cells to the inflamed site, the production of cytokines, and a respiratory burst. The respiratory burst, one of the first events in the cascade, results in the overproduction of reactive oxygen species (ROS). ROS is the name given to all of the metabolites of oxygen and their derived radical species, which include hydrogen peroxide, hydroxyl radical, reactive nitrogen species, and superoxide anion.

1.1.2. Superoxide Anion

In the respiratory burst oxygen is reduced to superoxide anion via the enzyme NADPH-oxidase. When the cell system is at rest, the NADPH-oxidase system also remains at rest. In inflamed cells, the soluble components of the cell, known as the cytosolic factors, are relocated to the plasma membrane, where enzyme assembly takes place (1). The superoxide is then produced by the activated enzyme complex as shown in the following reaction:

\[ \text{NADPH} + 2\text{O}_2 \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+ \]  
(Equation 1.1)

The superoxide anion has the potential to further react, producing species such as the hydroxyl radical that is a strong oxidant. Superoxide not only possesses oxidizing ability, as exhibited in
the oxidation of ascorbic acid, thiols, and phenols, but also a reducing agent as it reduces iron (III) and some quinones.

Reactive oxygen species are formed and degraded by all aerobic organisms, leading to either physiological concentrations needed for normal cell function, or an excess, best described as oxidative stress (2). ROS can serve as useful functions within organisms, as seen when looking at NO, which holds an important role in regulating blood pressure (3) as well as being a regulator of transcription factor activities and a determinant of gene expression (4). ROS also have negative effects including free radical-mediated oxidation of biomolecules, such as lipid, DNA, or protein. Biomolecule oxidation is associated with chronic diseases of stress such as inflammatory bowel disease, with diseases of aging such as cardiovascular disease, and with acute tissue injury such as ischemia (4). Therefore further understanding ROS as well as factors that provide protection from ROS would be useful.

1.2. Hypothesis

Quantitatively measuring radicals is a task that has several challenges due to the reactivity of radicals with other species. The analytical tool for measuring radicals is usually a compound that is oxidized or reduced by the radical with an accompanying change that can be detected, such as a color change. The redox sensitivity of the analytical tool means that it may be reactive with nonradical oxidizing or reducing agents. The goal of the work described in this chapter was to develop an alternative method for detecting superoxide production in the presence of polyphenolics (tannins), which are highly redox-active.
1.3. Cytochrome c for Detection of Superoxide

Cytochrome c is a heme protein that contains a centrally located iron ion. Previous work suggested that cytochrome c (Fe$^{3+}$) would be an ideal probe for measurement of superoxide due to its chemistry (5).

\[ \text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+} \quad \text{(Equation 1.2)} \]

When oxidized cytochrome c accepts electrons from a reducing agent, a color change occurs which can be detected spectrophotometrically. Cytochrome c is non-toxic to a cell based system and is a readily available agent, making it a preliminarily ideal probe. The cytochrome c reduction assay has been well established and has been previously used in detecting superoxide anions in mammalian cells (2). Cytochrome c has many advantages, but it does not work well in our system because it is reactive with polyphenols.

\[ \text{(Cyto c) Fe}^{3+} + \text{O}^\cdot \rightarrow \text{(Cyto c) Fe}^{2+} + \text{O}_2 \quad \text{Abs @ 550nm} \quad \text{(Equation 1.3)} \]

1.4. Luminol for Detection of Superoxide

1.4.1. Luminescence

Luminescence, also known as light emission, occurs when a molecule in an excited state relaxes back to its ground state. There are several types of luminescence in which their names are directly linked to the source of energy used to reach the excited state. For example, chemiluminescence uses energy produced by a chemical reaction. In chemiluminescence the chemical reaction produces energy at about 300 kJ mol$^{-1}$ and 120 kJ mol$^{-1}$ for blue and red
emission, respectively (6). The energy induces the transition from the ground state to the excited electronic state. Once the electron returns to its ground state, accompanied by the emission of a photon, chemiluminescence is observed.

1.4.2. Luminescence of Luminol

The luminescence of luminol has been used as a probe for measuring superoxide production by mammalian cells (9). Luminol penetrates the cell membrane (9); therefore, its chemiluminescence represents both intracellular and extracellular reactive species. When luminol reacts with an oxidizing agent, it is oxidized to the electronically excited aminophthalate anion. As it relaxes back to its ground state, photons are released and are detected by a photomultiplier tube within the luminometer (6). The luminol reaction requires a base and a strong oxidizing agent such as hydrogen peroxide or superoxide. We postulated that luminol would not be oxidized by polyphenols, and so there would not be interference from polyphenols in a luminol-based method for measuring superoxide.

Luminol alone may not yield sufficient signal (7), and therefore, enhancers of luminol emission have been developed. The enhancer is used to increase sensitivity of the chemiluminescence assay, making it possible to detect lower concentrations of superoxide anion. Many metal ions increase the rate of the luminol reaction, including Cu(II), and most commercial luminol based enhancers contain sodium orthovanadate (7). Our initial experiments focused on developing a controlled method for determining superoxide production using luminol, which would then be extended to measure superoxide produced by rabbit monocyte cells along with the addition of different polyphenolics. We first confirmed that our luminometer worked by observing the reaction of luminol with hydrogen peroxide in the presence of copper sulfate and
the presence or absence of polyphenolics. We then established an enzymatic method for producing low levels of superoxide using xanthine and xanthine oxidase. We confirmed enzyme activity by following xanthine oxidation to uric acid spectrophotometrically and then measured superoxide with luminol and an enhancer. Finally, we applied the luminol assay to a mammalian cell system that was induced to produce superoxide by the inflammatory cascade.

1.5. Methods

1.5.1. Reagents

For preliminary assays, reagent grade luminol (Sigma, CAS# 521-31-3) was dissolved using concentrated NaOH. H₂O₂ was used as the 30% commercially available solution (Fisher). CuSO₄ was dissolved in Nanopure water, yielding a concentrated solution. Pentagalloyl glucose (PGG) was provided by Yumin Chen (8) and was dissolved in Nanopure water at concentrations ranging from 1.5 mg/mL to 6.0 mg/mL. For xanthine oxidase experiments, a 0.1 M glycine, pH 9, buffer solution containing 1 mM EDTA and 1 mM luminol was prepared. Xanthine (Sigma) was dissolved in the buffer at a concentration of 0.05 mM. Both microbial (X-2252) and bovine milk (X4500-5UN) xanthine oxidase were purchased from Sigma and dissolved in or diluted into the buffer. The cellular luminescence enhancement system Diogenes kit (Atlanta, GA; www.nationaldiagnostics.com; Product # CL-202) was prepared as directed.

1.5.2. Luminometer Validation

Luminol was dissolved in the well of a 24-well plate with NaOH by placing a very small amount of luminol in the well and adding 50 µL of NaOH to the well initially and then adding more to reach a final reaction volume of 100 µL. Hydrogen peroxide (2 µL) was added to the
well followed immediately by CuSO$_4$ (1 µL). The plate was then promptly placed in the microplate reader (Synergy HT Bio-Tek) and read every 30 sec to determine luminescence values. When analyzing the effects of PGG, the phenolic was added last (9).

### 1.5.3. Enzyme-generated Superoxide

The oxidation of xanthine was carried out by combining 100 µL of xanthine oxidase (7.7 ng/µL for the bovine enzyme) and 1 mL of 0.05 mM xanthine with the a 0.1 M glycine, pH 9, buffer to give a final reaction volume of 1.5 mL. The increase in absorbance at 290 nm was measured spectrophotometrically over 5 minutes. The rate of reaction was then calculated using the extinction coefficient of uric acid (10), and the result was multiplied by 2 mol O$_2^-$ following the assumption that for every 1 mol xanthine oxidized, 2 mol of O$_2^-$ are produced. Daily the same xanthine oxidase was diluted 100-fold with the prepared buffer, and 100 µL of that diluted enzyme was mixed with 500 µL 0.05 mM of xanthine, 100 µL of Diogenes solution, and brought to final volume of 665 µL in a 24-well plate. Chemiluminescence was measured every 30 sec over 5 minutes. For enzyme activity experiments with PGG, PGG was dissolved in water, its concentration determined spectrophotometrically, and it was added to the enzyme and buffer before adding the substrate.

The cells used in this assay were isolated in the same matter as described previously by Jeffers (11).
1.6. Results and Discussion

The expected blue luminescence (12) was observed when hydrogen peroxide, copper sulfate, and luminol solutions were combined as described above. When PGG was added to the assay, it did not change the luminescence of hydrogen peroxide/copper sulfate mixture (Figure 1.1), showing lack of inhibition or stimulation in the assay. In addition, PGG, in the absence of hydrogen peroxide, did not cause luminescence, indicating that the phenolic itself did not oxidize luminol to the excited, chemiluminescent state. The obtained luminescence values ranged from 0 to 200 luminescence units, but most previously reported values for luminescence are 100 times greater than these values (13). The plate reader setting for sensitivity can be set to an integer between 25 to 255 depending on the strength of the signal that the assay produces. For this assay sensitivity was set to 25 after trying a range of other values. At higher settings the value range of readings was scattered with some wells yielding no values at all. The setting of 25 gave the best ordered luminescence response both graphically and numerically, however, values were still lower than expected. It is not completely clear why such low luminescence values were detected, but we decided to attempt the superoxide assay despite this apparent sensitivity limitation of our instrument.

We planned to calibrate luminescence with superoxide production by using the oxidation of xanthine to produce superoxide, a reaction that can be quantitatively monitored spectrophotometrically. Xanthine is oxidized to by XO (xanthine oxidase), a molybdenum (Mo) containing enzyme. The Mo is reduced in the first phase of the reaction, and uric acid is made. The oxygen in the uric acid comes from water, and two protons are then released. Mo is then re-oxidized by oxygen, with the oxygen reduced to superoxide. The produced superoxide is then protonated to yield hydrogen peroxide (Equation 1.4)(14). The reaction was monitored
spectrophotometrically to observe uric acid production and with chemiluminescence to observe superoxide production.

\[
\text{xanthine} + \text{H}_2\text{O} \xrightarrow{\text{Mo (}} +6 \text{)} \text{uric acid} + 2\text{H}^+ + 2\text{O}_2
\]

(Equation 1.4)

Microbial xanthine oxidase and bovine milk xanthine oxidase are both commonly used enzymes that have been extensively studied and play a key role in purine catabolism (15). When microbial xanthine oxidase was combined with xanthine, absorbance at 290 nm increased in a linear fashion over five minutes with relative absorbance values ranging from 1 to 1.6 (Figure 1.2a). The same xanthine oxidase and fresh xanthine was combined with the Diogenes enhancer in the luminol-containing buffer to be evaluated via luminescence, but no signal was detected over 20 minutes (Figure 1.2b). The sensitivity for luminescence was set to 150 due to the weakness of signal.
To evaluate why we could not detect superoxide, we tested various concentrations of xanthine, luminol, buffer, and enzyme. Ultimately we tested an alternative source of enzyme, from milk. Bovine milk xanthine oxidase yielded similar spectrophotometric results as the microbial xanthine oxidase, although the absorbance values were slightly lower ranging from 1 to 1.2 relative absorbance units (Figure 1.3a). The calculated rate based on the spectrophotometric data was 6.4 nmol O$_2^\cdot$ min$^{-1}$ µg protein$^{-1}$. Chemiluminescence results were significantly different from those obtained when microbial xanthine oxidase was used (Figure 1.3b). Luminescence values followed a curved pattern, with an initial reading of about 15,000 luminescence units and decreasing to zero over 20 minutes (Figure 1.3b). We calculated the area under the curve during the 20 min reaction, and using the rate determined spectrophotometrically, we established that 25 area units corresponded to production of one pmole of superoxide. We concluded that the microbial xanthine oxidase was contaminated with superoxide dismutase, and thus although the enzyme effectively oxidized xanthine, superoxide was destroyed as it was produced and could not be detected. All further experiments were conducted using the bovine milk xanthine oxidase.

These results show that the enhancer system made luminol a sensitive detection agent for superoxide, therefore we proceeded to apply this assay to our cell based system. Cells were isolated, primed with LPS (lipopolysaccharide) and stimulated with PMA (phorbol 12-mysristate 13-acetate) to initiate the superoxide burst (11). Luminol and enhancer were added to the cells along with the PMA at levels suggested by the commercial literature supplied with the enhancer. Very low levels of luminescence were observed over a two hour time frame (Figure 1.4). The kinetics of production of superoxide by the cells as detected with luminescence were consistent with results obtained with cytochrome c to detect superoxide, but the luminescence values were
very low, and moles of superoxide produced could not be calculated. We attempted to use the method to determine the effects of polyphenolics on the superoxide burst. LPS was added first and then PGG was added to the wells after plated cells adhered to the plate during a 24 hour incubation. When the PGG was added to the cell system, luminescence data indicated that PGG might suppress the superoxide burst (Figure 1.4).

We postulated that PGG might either inhibit the superoxide burst, or might simply scavenge superoxide as it is made by the cells. As previously shown in equation 4, superoxide is a byproduct of xanthine and xanthine oxidase chemistry. Therefore this enzyme-based system was used to test the possibility that PGG scavenges superoxide. The spectrophotometric data indicated that 7.7 μM PGG caused a slight lag in the oxidation of xanthine, with a subsequent apparent rate higher than that obtained in the absence of PGG (~25 nmoles O₂⁻/min/ug protein) (Figure 1.5a). The luminescence data showed that PGG did slow the rate of production of superoxide by the xanthine oxidase (Figure 1.5b). The observation that PGG does not inhibit the oxidation of xanthine, but does retard the rate of accumulation of superoxide, indicates that PGG is as a scavenger of superoxide. Thus one possible role of PGG in biological systems is to scavenge superoxide produced either by normal physiological processes such as the superoxide burst phase of inflammation, or by metabolic side reactions such as escape from mitochondrial electron transport.

In conclusion, we developed an alternative to the cytochrome c method to measure superoxide production by PMBC using luminol as the probe. We demonstrated that PGG did not interfere directly with the luminescence reaction. We found that the method was insensitive when applied to our cell system and believe that considerable work would be required to increase its sensitivity to make detection of superoxide production by monocytes feasible. Despite the
limitations of the method, we did obtain results that suggested a possible mechanism of action for PGG as a scavenger of superoxide.
Figure 1.1 *Luminescence of luminol.* Luminescence of luminol plus hydrogen peroxide in the presence of CuSO₄ and/or PGG. Data were collected for 10 min after adding peroxide, and the area under the curve (AUC) calculated with GraphPad software. Average data from three assays and standard deviations are shown. (Luminol: LumTann.2.16.06A)
Figure 1.2a  *Oxidation of xanthine using microbial XO*. Relative absorbances were determined by dividing absorbance values by the initial absorbance to normalize data. (UV-Vis Abs Data_Graphs_Analysis: Bacterialxo3_3.23.06)
Figure 1.2b Microbial XO luminescence. Luminescence produced by oxidation of xanthine using microbial xanthine oxidase. (X XO_Diogen: 3.23.06)
**Figure 1.3a Oxidation of xanthine using bovine milk XO.** Oxidation of xanthine using bovine milk xanthine oxidase with a reaction rate of 6.4 nmol O$_2$·/min/ug protein. Relative absorbances were determined by dividing absorbance values by the initial absorbance to normalize data. (UV-Vis Abs Data_Graphs_Analysis: Milkxo2_4.18.06)
**Figure 1.3b** Bovine milk XO luminescence. Luminescence produced by oxidation of xanthine using bovine milk xanthine oxidase. (XO Plate Reader Data_Graphs_Analysis: 100µL XO 4.18.06)
Figure 1.4 *Normalized luminescence.* Normalized luminescence produced by LPS/PMA-stimulated cells with and without the addition of tannin (PGG). (XO Plate Reader Data_Graphs_Analysis: tannin5.18.06 and notannin5.18.06)
Figure 1.5a Oxidation of xanthine with PGG. Oxidation of xanthine using bovine milk xanthine oxidase with the addition of PGG and a reaction rate of about 25 nmol O$_2^-$/min/ug protein. Relative absorbances were determined by dividing absorbance values by the initial absorbance to normalize data. (UV-Vis Abs Data_Graphs_Analysis: Milkxo2_PGG5.15.06)
Figure 1.5b  *Superoxide production with PGG.* Superoxide produced by oxidation of xanthine using bovine milk xanthine oxidase in the presence of various amounts of PGG. Each point is the average of 3 determinations. Standard errors are smaller than the symbols used.

(X_XO_Diogen: 5_15_06A_aeh)
1.7. Chapter 1 References


2. Superoxide Scavenging by Polyphenols

2.1. Introduction

2.1.1. Reactive Oxygen Species Damage

The formation of ROS in biological systems has been linked to several degenerative diseases such as cardiovascular disease, cancer, Crohn’s disease, and many others (9). An uncontrolled production of ROS and an unbalanced mechanism of antioxidant protection may cause the onset of these diseases and accelerate aging. ROS are either a by-product of typical mitochondrial metabolism or a constituent of the inflammatory pathway, as discussed in the previous chapter. Damage is primarily due to the production of hydroxyl radical, which is formed by the Fenton reaction. First superoxide reacts with H⁺ yielding hydrogen peroxide (Equation 2.1).

\[ 2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \]  (Equation 2.1)

Hydrogen peroxide is a long lived oxidant that may then produce the highly-reactive hydroxyl radical by way of the Fenton reaction (Equation 2.2). ROS such as hydroxyl radical lead to oxidative alterations in the cellular membrane or intracellular molecules, however, they are removed by antioxidant defense mechanisms (1).

\[ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^- \]  (Equation 2.2)

2.1.2 Antioxidants vs. Anti-Inflammatory Agents

In general, antioxidants inhibit production of ROS or quench radical species, while anti-inflammatory agents repress individual biochemical steps in the inflammatory cascade (1). Antioxidants serve to counteract ROS attacks and protect the organism from ROS damage (1). Therefore, there is an increasing interest in substances that exhibit antioxidant properties in both the food and pharmaceutical worlds.
2.1.3. Tannins

2.1.3.1. History of Tannins

Tannins have been studied for both their antioxidant and anti-inflammatory properties. They are a family of naturally-occurring polyphenols that characteristically precipitate proteins (2). They tend to form insoluble, cross-linked complexes with proteins and have been used in many different arenas. Tannins were originally used to tan animal hides into leather, hence the name tannin. This process occurred via the precipitation of the proteins found in the skin of the animals such as collagen (3). Another characteristic of tannins is their potency as metal ion chelators. Ion chelators in general serve to bind with high affinity to metals such as iron or manganese (4). Finally, because they are phenolics, tannins are excellent radical scavengers and may be useful biological antioxidants.

2.1.3.2. Classes of Tannins

Tannins can be divided into two different classes in which have biological significance to humans; condensed and hydrolyzable tannins (2). Condensed tannins, or proanthocyanidins, are polymers of two or more flavonoid units, conjoined by carbon-carbon bonds, not allowing for hydrolysis to occur. Condensed tannins can be degraded to pigments known as anthocyanidins. An example of a condensed tannin would be procyanidin found in Sorghum grain. Hydrolyzable tannins maintain a core polyol, usually glucose, with galloyl groups derived from shikimic acid esterified to the core (4). These tannins are hydrolyzed by weak acids or bases that therefore yield carbohydrates and phenolic acids. A common example of a hydrolyzable tannin is pentagalloyl glucose (PGG), which is often referred to as a gallotannin. Both examples for the condensed and hydrolyzable tannins are represented in Figure 2.1.
2.1.3.3. Tannin Consumption

Tannins are found throughout the plant kingdom and are consumed daily by humans (5). Some of the most commonly consumed tannins are found in green and black tea, red wine, apple juice, and several fruits, most notably berries. Uptake of tannins varies due to daily diet and availability of produce (5). For instance, in countries in and around the Middle East where availability of tannin containing foods is higher, consumption is higher than that of North America. It has been reported that tannin consumption in India ranges from 1.5-2.5 g/day, depending on the region, and about 1g/day within the USA (5).

There are both pros and cons associated with tannin consumption. High levels of dietary tannins have been reported to limit protein digestibility as well as reduce the uptake of essential metals (6). Most of the direct effects of dietary consumption of tannins are thought to be linked to how they bind dietary constituents in the digestive tract. For example, formation of tannin-protein complexes in the GI tract is thought to prevent absorption and utilization of dietary protein.

2.1.3.4. Tannins as Radical Scavengers

Reports of both antioxidant and anti-inflammatory activity of tannins suggest a likely mechanism for their role in disease prevention. Most tests of radical scavenging activity of tannins have been made with nonbiological model radicals such as the colored ABTS radical cation (7, 8). We proposed to test scavenging of biologically relevant radicals more directly.

ROS play a key role in the inflammatory cascade, with ROS present at every stage of the physiological defense of inflammation. As discussed in chapter 1, reactive oxygen species have an unstable nature and can form both beneficial and harmful products in cell systems. There is a
connection between diseases involving inflammation and ROS, for example the pathology of IBD (inflammatory bile disease) is frequently linked to oxidative activity in the gastrointestinal tract (9). ROS generating enzymes are in place to box in the area of inflammation and inhibit the spread of damage. This allows the protection of the remaining healthy tissue from the release of bactericidal gases. Radicals, such as O$_2^-$ and NO, kill invading bacteria but also have the capacity to react with other species and produce more toxic agents, such as OH$^-$ radical and hydrogen peroxide (9). Production of ROS must be controlled within the inflammatory cascade.

2.2. Hypothesis

Superoxide radical is the primary radical product of both leaky mitochondrial electron transport and of the inflammatory cascade. Being able to control superoxide production within biological systems could be an important addition to obtaining needed equilibrium of ROS. Therefore testing the superoxide scavenging ability of several phenolics could lead to insight into the anti-oxidant and anti-inflammatory properties of these tannins. With that said, it was our goal to examine the effectiveness of PGG and several grape seed extracts as superoxide scavengers.

2.3. Methods

2.3.1. Reagents

Nitro blue tetrazolium (NBT; N-6876), phenazine methosulfate (PMS; P-9625), reduced β-nicotinamide adenine dinucleotide (NADH; N6005), trizma hydrochloride (Tris-HCl; T-3253), quercetin (Q-0125), and rutin (R-9000) were obtained from Sigma-Aldrich (St. Louis, MO). Both NBT and NADH were dissolved in 16 mM Tris-HCl, pH 8.0, while PMS was dissolved in Nanopure water. Pentagalloyl glucose (PGG) was provided by Yumin Chen (10). The reagent
grade grape seed fractions were obtained from “OenoConsulting”, Chisinau, Republic of Moldova. These preparations were obtained by extracting grape seeds with water-ethanol solutions and then fractionating the extract (Andrei Prida, personal communication). The material provided as “Standard” (ST) was the ethanol-water extract of the grape seeds. That extract was evaporated under reduced pressure to remove ethanol, filtered, and brought back up to the initial volume with water to yield the fraction “Water-Soluble” (WS). The insoluble material from that step was redissolved in ethanol to yield the fraction “Alcohol-Soluble” (AS). The fraction identified as “Pharmaceutical” (PH) was obtained by purifying the initial standard extract on a polystyrene-based adsorbent column. The exact compositions of these fractions were unknown.

2.3.2. Superoxide Production and Scavenging

The grape seed extracts were prepared at a concentration of 300 µg/mL in methanol. AS was dissolved in aqueous methanol (50:50, v/v) to overcome its poor solubility properties. Superoxide anion radicals are produced when PMS reduces NADH, and the superoxide immediately reacts either with NBT to form a colored species, or reacts with the test scavenger (11, 12). The reaction was started by adding 38 µM NADH to mixtures containing 50 µM NBT, 10 µM PMS and varying concentrations (0-20 µg/mL) of scavenger, which was brought to a total volume of 3.0 mL with the addition of 16 mM Tris-HCl buffer pH 8.0. The initial rate of formation of NBT was determined at 560 nm over the first 20 s of each reaction, and the % scavenging was calculated by comparing the rate of formation of NBT in samples with scavenger to the rate in samples without scavenger. Each concentration of each fraction was assayed three times. The mixture without sample was the control and mixture without addition of PMS was used as the blank. The IC50 values were determined by plotting % scavenging as a function of
grape seed fraction concentration and fitting the data to a hyperbola with GraphPad Prism 4 software.

### 2.3.3. Validation

The two flavonoids, quercetin and rutin, were used to validate the assay. We compared scavenging percentages for these two compounds to published values (12).

### 2.4. Results and Discussion

In the NBT based system, superoxide anion is the product of PMS oxidation by oxygen. Superoxide reduction of NBT is observed colorimetrically, by the formation of a blue/purple color with strong absorbance at 560 nm. Superoxide generation is maintained by NADH recycling of the PMS as shown in Figure 2.2. In the presence of superoxide scavengers, superoxide reduces the scavenger rather than the NBT, so color formation is suppressed. We monitor the initial rate of formation of reduced NBT with inhibition of this formation being the basis of determining superoxide scavenging ability of flavonoids.

Modifications to the published assay (12) were developed during our optimization process. Zou et al. reported that PMS should be used to initiate the reaction, but we found that NADH was more effective. We also found that the recommended concentration of NADH was twice as high as necessary. In our optimized method we used 38 µM NADH added after the PMS, NBT and scavenger.

For validation purposes two flavonoids were chosen from the list of compounds provided in the Zou et al. paper (12). We found that both compounds, quercetin and rutin, exhibited scavenging characteristics very similar to those reported in the literature (12). Addition of quercetin decreased formation of NBT in a linear fashion (Figure 2.3). Rutin yielded the same
Percent scavenging was calculated by comparing the absorbance of the sample at each concentration of scavenger to the absorbance of the control sample. The IC50 values for quercetin and rutin were 1.1 ± 0.2 µg/mL and 14 ± 4 µg/mL. In comparison, Zou et al. reported that rutin was about three times more effective than quercetin. Our 14-fold difference could be the result of the improvements we made in the analytical procedure, differences in flavonoid purity, or methods of fitting the data.

After optimization was complete, PGG was tested in the system to evaluate its superoxide scavenging ability. As discussed in chapter 1, data from our cell-based assay for inflammation suggested that PGG was an effective superoxide scavenger. The concentration of PGG was increased from 0 µg/mL to 115 µg/mL in our experiments. The expected results were that as the PGG concentration was increased the absorbance values at 560 nm would decrease in a linear fashion. Figure 2.4 shows that as concentration of PGG increased, absorbance varied little at 560 nm. We ultimately realized that the results were a consequence of interference chemistry between PGG and NBT (13). Phenolics can be oxidized by oxygen in a 2 electron, radical-mediated pathway. The first oxidation yields a semiquinone and the second step quinone. If oxygen is the oxidizing agent, and the pathway goes by consecutive one electron steps. The product of each step is superoxide, therefore yielding a more intense formation of color as illustrated in Fig. 2.2. However, not all phenolics follow the one electron oxidation pathway and may react differently within the system.

When evaluating the chemistry presented in Figure 2.2, one would suggest that there be an increase in reduced NBT if PGG produced superoxide via oxidation. We saw no net change in NBT color formation in the presence of varying amounts of PGG. We therefore postulated
that a balance occurs between superoxide production and scavenging with complex phenolics such as PGG. Therefore this assay is not ideal for measuring superoxide scavenging by PGG.

In chapter 1, we described the interference between polyphenolics and cytochrome c that prevented effective measurement of superoxide concentration in our polyphenolic-containing cells. Here we describe interference by polyphenolics with the NBT assay for superoxide. Similar problems with indirect measures of superoxide and superoxide scavenging have led other workers to resort to spin-trap EPR measurements on cell preparations (14). We plan to explore similar direct methods for measuring superoxide in future experiments on our cell systems.

The assay did provide a foundation in which other tannins that are not able to form superoxide directly, could be analyzed for their ability to scavenge superoxide. OenoConsulting supplied reagent grade grape seed extract that could be analyzed. Grape seed extract (GSE) is known to be a rich source of proanthocyanidins. Proanthocyanidins have been associated with several health benefits including anticancer effects, increasing intracellular vitamin C levels, and decreasing capillary permeability and fragility (15, 16). It has also been noted for its antioxidant and superoxide scavenging properties. GSE has been marketed as a dietary supplement, so that understanding its effects in more detail would be favorable (15).

The grape seed extract samples were assayed as described above for the flavonoids. As the amount of extract was increased the superoxide scavenging percentage also increased. The average percent scavenging for each extract is displayed in Figure 2.5. In order to compare data for various extracts, IC50 values were determined. The IC50 values for superoxide scavenging increased in the following order: ST<WS<PH<AS. When IC50 values were compared for all the samples for their different types of scavenging ability, the superoxide and hydroxyl radicals followed very similar patterns with increasing values, while peroxyl and ABTS had very little
difference from extract to extract (Figure 2.6). A form of this chapter has been submitted for publication and is provided in Appendix 1 of this thesis.
Figure 2.1 Structure of PGG and procyanidin.
Figure 2.2 *NBT reduction assay chemistry.*

\[
\begin{align*}
\text{NADH} & \quad \text{PMS ox} \quad \text{O}_2^- \quad \text{NBT ox}^- \\
\text{NAD}^+ & \quad \text{PMS red} \quad \text{O}_2 \quad \text{NBT reduced colored}
\end{align*}
\]
Figure 2.3 *NBT reduction assay with addition of Quercetin.* Absorbance at 560 nm with the addition of increasing concentrations of quercetin. (GSEDATA: Quercetin6.20.06)
Figure 2.4 *NBT reduction assay with addition of PGG.* Absorbance at 560 nm with increasing concentrations of PGG from 0 µg/mL to 115 µg/mL. (GSEDATA: Varying PGG6.2.06)
Figure 2.5 *GSE percent scavenging.* Average superoxide scavenging percentage of grape seed extracts ST, AS, PH, WS, and ST. (GSEDATA: Summary7.21.06)
Figure 2.6 *GSE IC₅₀ values.* IC₅₀ values for superoxide, hydroxyl, peroxyl and ABTS radical scavenging. Data for hydroxyl, peroxyl and ABTS are described in the publication provided in Appendix 1. (GSEDATA: Summary7.21.06)
2.5. Chapter 2 References


3. TNF-α Production by Monocytes in the Presence and Absence of Polyphenols

3.1. Introduction

Inflammation is a word commonly used to describe the physiological response the body gives to injury or tissue destruction. Disruption of this physiological control mechanism of defense yields the foundation of the pathology of many diseases. The inflammatory response is categorized as either acute or chronic depending of the duration and kinetics of the reaction. It has been suggested that acute inflammation is associated with rapid physiological mechanistic activity, while chronic inflammation is more associated with organized repair systems (1). Chronic inflammation may result in symptoms that are never resolved due to continuous inflammatory reactions and disruption of physiological states. Several diseases are associated with this level of persistency including rheumatoid arthritis, atherosclerosis, inflammatory bowel disease (IBD), and Alzheimer’s disease.

3.1.1. Inflammatory Cascade

The series of events involved in the inflammatory cascade is not completely understood due to its complexity. However it is suggested to be a chain of events that occurs in response to the toxic and lethal effects of bacterial endotoxins, such as LPS (lipopolysaccaride)(2), and that includes a feedback mechanism that facilitates regulation. The inflammatory cascade initially causes vasoconstriction, which then causes an increase in calcium activated nitric oxide synthase (NOS) to release radical NO. The NO radical has the ability to relieve the stress of vasoconstriction. Once the vessel is open, the reperfusion phase is activated which in turn causes
an increase in \( \text{O}_2^\cdot \) concentration. The superoxide will react to yield peroxynitrate, hydroxyl radical, and other bacteriocidal radicals. These ROS recruit more phagocytic cells to the site and increase bactericidal activity by way of the respiratory burst in which additional superoxide is generated by a membrane-bound NADPH oxidase (1). All of the ROS that are released in this process interact with resistant cell types and cause an increase in the release in inflammatory cytokines, metalloproteinases, and adhesion molecules all of which serve as types of mediator of the inflammatory process (1).

### 3.1.2. Cytokines TNF-\( \alpha \) and IL-1

There have been several supporting reports that link cytokine production to inflammatory diseases. Cytokines are proteins that work intercellularly to mediate immunologic and nonimmunologic biological functions. Insufficient production of cytokines can lead to states of immunosuppression, while overproduction can lead to severe shock or autoimmune diseases. IL-1 (interleukin-1) and TNF-\( \alpha \) (tumor necrosis factor-alpha) are among the most commonly studied cytokines, and are said to be potent inducers of prostaglandins, thromboxanes, and other biomolecules (3). TNF-\( \alpha \) and IL-1 are both elevated during inflammation.

TNF-\( \alpha \) has been linked to necrosis, or the accidental death, of tumor cells in a less orderly fashion than apoptosis. TNF-\( \alpha \), also known as cachectin, causes cellular proliferation, differentiation, inflammation, tumorigenesis, and viral replication and has also been implicated as the primary agent responsible for inducing shock and other related physiological syndromes (3). TNF-\( \alpha \) is mainly produced by macrophages in response to LPS and other bacterial products. Being able to monitor TNF-\( \alpha \) secretion is beneficial to the pharmaceutical world due to its pro- and anti-carcinogenic properties (4). High dosage of TNF-\( \alpha \) at a localized site has been said to
selectively demolish tumor blood vessels yielding great anti-cancer actions, however if produced at a chronic level, TNF-α may act as a tumor promoter (5). TNF-α serves as a cell signaling molecule in the inflammatory cascade (3). Therefore TNF-α serves an excellent probe to monitor cell reactions within the cascade allowing us to directly test our proposal that polyphenolics are potential anti-inflammatory agents.

3.1.3. PMBCs (Peripheral Mononuclear Blood Cells)

A source of cells was required to allow us to develop an ex vivo system for assessing the anti-inflammatory activities of various tannins and their affect on TNF-α production. We chose to use PBMCs from a mammalian source. Peripheral mononuclear blood can be collected consistently throughout the duration of the animal’s life. The mammal of choice was a rabbit for as long as the rabbit is in good health when the blood is drawn, results can be compared across experiments.

PBMCs are made up of between 25-35% monocytes/macrophages, 65-75% lymphocytes, and less than 1% neutrophils, eosinophils, and basophils (6). Because cells other than monocytes/macrophages may produce cytokines and other inflammatory stimuli, it is important to include the entire white blood cell population rather than just enriched subpopulations. This also serves as a quicker method of isolation. Once cells have been obtained, they are then primed to enhance the cellular functions and increase phagocytosis. The priming of the cells takes place in the presence of an added bactericidal agent such as LPS. With this addition cytokines are released as described above.

LPS has been shown to up-regulate complement receptors and adhesion molecules to allow cells to adhere to Teflon or serum-coated glass (7). Adherence is an essential part of the
immune response, and it has been suggested that it contributes to superoxide production in vitro (8). In vivo, monocytes migrate from the blood stream into the inflamed tissue to differentiate into macrophages, and adherence is a necessary part of this process. Based on the literature, we chose to isolate PBMCs from rabbit blood and to culture them for up to two days in a CO2-enriched atmosphere in tissue culture plates that promote adherence. We chose to prime the cells with LPS before measuring inflammation by monitoring TNF-α production via an ELISA assay.

3.1.4. ELISA (Enzyme Linked Immunosorbant Assay)

ELISA assays are commonly used to determine the concentration of biomolecules in unknown samples due to the high specificity and sensitivity of the method (9). There are two types of commonly used ELISA assays, sandwich and competitive. The sandwich ELISA assay is a fast way to determine the absolute amount of antigen if purified standards are available to compare against. This assay requires two purified antibodies in which an immobilized primary antibody (capture antibody) binds the antigen to a plate surface and the secondary antibody (detection antibody) then binds to the stationary antigen thus forming a sandwich complex. Detection enzyme is then bound to the sandwich for quantitation of antigen. The competitive ELISA differs in that it does not require exact matches of antibodies and that the primary antibody does not have to be purified. In order for competitive ELISA to be used, one of the reagents used must be conjugated to the detection enzyme. The sandwich form of the assay is more appropriate to use if antibodies are available for desired antigens. TNF-α has been measured using sandwich ELISA in several previously published works and commercially available capture and detection antibodies are available, hence we chose the sandwich ELISA for our investigation (10, 11).
3.2. Methods

3.2.1. Reagents

All glassware was sterilized by baking in a drying oven for 24 hours, and other items were sterilized by autoclaving under standard conditions. Na₂EDTA (Sigma ED2P) was dissolved in water at 0.1 mM and filter sterilized with vacuum filtration using Corning disposable bottle top filters with 0.22 μm polyethersulfone membranes. To obtain Histopaque with a density of 1.10 g/mL, Histopaque 1.083 g/mL and Histopaque 1.119 g/mL were mixed in equal volumes. All Histopaque was purchased sterile from Sigma. Solutions were mixed under the sterile hood and stored in a sterilized glass bottle at 4°C. RPMI 1640 medium, without L-glutamine, phenol red, or sodium bicarbonate, was purchased from Caisson Laboratories (RPMI-013P). The medium was prepared by Molly Jeffers and was reconstituted with Nanopure water and supplemented with 2 g/L of sodium bicarbonate, 2 mM L-glutamine, 100 units/mL of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (Invitrogen, 10082-139). The medium was filter-sterilized and poured into a one-liter media bottle with a screw top and stored at 4°C. Hank’s balanced salt solution (HBSS) without calcium, magnesium, or sodium bicarbonate was purchased from Sigma (H4385) as a 10x stock, which was diluted ten times with the addition of sodium bicarbonate at a concentration of 0.35 g/L. Sterilization was done in the same manner as media, however, it was stored at room temperature in the sterile hood. Trypan blue (Sigma T-6146) was dissolved at 4% by weight in PBS buffer, pH 7.8, with 0.1 mM EDTA, and stored at room temperature in the sterile hood. LPS from *E.coli*, (Sigma L-2880), was made by Molly Jeffers by dissolving the solid in PBS buffer, pH 7.8, supplemented with 0.1 mM EDTA, at a concentration of 0.25 mg/mL for storage in the -20 freezer. It was then diluted to
1x10^{-4} \text{ mg/mL} \text{ for use with the cells. Rabbit TNF-}\alpha \text{ ELISA kit reagents were purchased from BD Biosciences Pharmingen and consisted of assay diluent, coating buffer, wash concentrate, stop solution, capture antibody (purified goat anti-rabbit TNF polyclonal antibody; 1\textsuperscript{o} antibody), streptavidin/HRP (horseradish peroxidase), detection antibody (biotinylated mouse anti-rabbit TNF monoclonal antibody; 2\textsuperscript{o} antibody), and substrates A (hydrogen peroxide) and B (tetramethyl benzidine, TMB). Assay diluents were modified by adding additional bovine serum albumin (BSA, Sigma A-6003) at a final concentration of 1\% (w:v), however, this was not added to the coating buffer.}

3.2.2. PMBCs Isolation

To obtain PBMCs, approximately 50 mL of rabbit blood was collected in Na\textsubscript{2}EDTA supplemented Falcon tubes (50 mg Na\textsubscript{2}EDTA in a 50 mL tube) with a 21 gauge butterfly needle. The blood was then diluted with sterile-filtered HBSS without calcium and magnesium at a ratio of 1:2, (v:v, blood:HBSS), and split into several Falcon tubes allowing room for the Histopaque. Histopaque (density = 1.10 g/mL) was slowly layered underneath the diluted blood at a ratio of 4:3 (v:v, blood:Histopaque) using a 10.2 cm long needle. Samples were centrifuged in a swinging bucket centrifuge (IEC Centra-8) at room temperature for 30 min at 700 x g. The top layer, plasma, was carefully removed and discarded. Using a fine tipped sterile transfer pipette, the next layer, containing the mononuclear cells, was removed and transferred to an autoclaved 50 mL Falcon tube. The cell suspension was then centrifuged for 30 min at room temperature and 700 x g, to pellet the cells. The supernatant was removed, and the cell pellet was resuspended in approximately 12 mL HBSS. The samples were centrifuged for 30 min at room temperature and 700 x g to pellet the PBMCs. The cells were then washed two times with HBSS by resuspending the cells in HBSS, and then centrifuging the cell suspension at 700 x g, room
temperature, for 20-30 min or until the supernatant was clear. Finally, the cells were resuspended in RPMI 1640 medium at a ratio of one volume of medium to two volumes of blood collected. The medium was warmed in a water bath to approximately 37°C, prior to use. Cells were then counted on a hemocytometer, and viability was determined with trypan blue staining. Cells were diluted 1:20 with the 4% trypan blue stain, and viable cells were identified by their exclusion of the blue stain.

After counting was completed, cells were placed in a 24-well plate at a concentration of 6.0x10^6 cells/mL, using RPMI 1640 medium to achieve that concentration with a final volume of 500 µL/well. The plates were sealed in CO₂ (5-12%) producing bags (Becton, Dickinson and Co; BBL GasPak CO₂ Pouch Capnophilic System) and incubated for 24 hours at 37°C. During this time cells adhered to the bottom of the plate. After the first 24 hour incubation the supernatant was removed from each well, and fresh medium was added along with 25 µL of LPS (0.1 ng/µL) and varying concentrations of PGG with a total well volume of 500 µL. Plates were sealed in new CO₂ producing bags and incubated for an additional 24 hours at 37 °C. Cells and supernatant were then placed in individual micro-centrifuge tubes and centrifuged at room temperature at 9,300 x g for 10 minutes. The cell pellet was discarded, and the supernatant was placed in new sterile micro-centrifuge tubes to be stored at -80°C until ready for assay.

3.2.3. ELISA (Enzyme Linked Immunosorbent Assay)

All reagents in the ELISA kit and 96-well plate were taken from 4 °C and allowed to come to room temperature before being used. Capture antibody (1° antibody) was then diluted in the commercial coating buffer at a concentration of 8 µg/mL, and 100 µL was placed in desired wells of a 96-well ELISA plate. The plate was then sealed with a PCR-plate cover and pulsed five times in the Boekel Scientific tabletop shaker to assure that the reagent covered the surface
area of the well. The plate was then incubated at 4 °C for 24 hours. The contents of the plate were emptied using the Bio-Tek automated strip washer as described below. The automated strip washer was set to cycle 6-7 times with a 60 second soak between each wash. The strip washer was set to dispense 250 µL/well at a flow rate of one (150 µL/well/sec), at a height of 120 (15.240 mm), and primed before the wash began with 5 mL of wash buffer at a flow rate of 5 (500 µL/well/sec). The aspirator was set at 50 (6.350 mm) at a rate of 1 (5.0 mm/sec) with a final aspiration included. After washing, 100 µL of the BSA-amended assay buffer was added to each well. The plate was sealed, pulsed, and incubated at 4 °C for an additional 24 hours. The plate was washed using the Bio-Tek automated strip washer six times with 60 seconds of wash buffer incubation allowed between each wash, as described above. The plate was then tapped upside down to remove droplets, but not allowed to dry before 100 µL of the desired sample or the TNF-α standard was added to the wells. Unknown samples were comprised of aliquots of the supernatants from the cells, which were stored at -80 °C until needed for the assay. The samples were thawed slowly to room temperature and gently mixed before being added to the plate. The commercial TNF-α standard was dissolved in BSA-amended assay buffer to make a stock solution of 8,000 pg/mL and was then serially-diluted to yield concentrations of 8x10³, 4x10³, 5x10³, 2x10³, 1x10³, and 5x10² pg/mL. The plate was then sealed, pulsed, and incubated overnight at 4°C. The plate was washed an additional five times, and the detection antibody was diluted to a final concentration of 3 µg/mL in the BSA-amended assay diluent. The detection antibody was then added to the empty wells, sealed, pulsed, and incubated at room temperature for one hour. After the one hour incubation the plate was washed five additional times and tapped upside down but not allowed to dry. The streptavidin avidin/HRP was then diluted 1:5000 in the BSA-amended assay diluents, and 100 µL of the
enzyme was added to each well. The plate was sealed without pulsing at room temperature for 30 minutes. The plate was then washed seven times for the final wash. Substrates A and B were mixed 1:1 by volume and added to wells of the plate quickly. The plate was then immediately placed in the plate reader to measure absorbance at 652 nm for one hour.

3.2.4. TNF-α Optimization

Primary and secondary antibodies were tested at several different concentrations to determine the most ideal setting for slow yet detectable development. The TNF-α standard was tested at various concentrations to obtain a linear response. Problems with the assay led us to test all components of the kit for spurious HRP activity, to test various washing protocols, to test order of addition of reagents, and to test effective blocking of the 96 well plate with the assay diluent.

3.3. Results and Discussion

Optimization of the ELISA assay required several different troubleshooting experiments. The initial goal was to determine the best concentrations of the primary and secondary antibodies that would yield the most sufficient colormetric detection. This was done by evaluating TNF-α standards at varying concentrations of primary and secondary antibodies. The 1° antibody ranged in concentrations from 1 µg/mL to 12 µg/mL, while the 2° antibody ranged in concentration from 1 µg/mL to 6 µg/mL. The optimized concentrations were determined to be 8 µg/mL and 3 µg/mL for primary and secondary antibodies, respectively. Once this was determined several sets of standards were assayed to determine the reproducibility of this assay. During this time several other obstructions to the assay were observed and assessed to further to
eliminate the spurious results. These included incidental peroxidase activity, microbial contamination, and insufficient plate blocking, all of which will be discussed in more detail.

The same TNF-α standard concentrations were tested in replicate, yet there was a large amount of variability from one set of standards to the next. One proposed source of spurious results was that of incidental peroxidase activity. The presence of endogenous peroxidase activity within the system had the potential to catalyze substrates used to detect conjugated peroxidase yielding high absorbance values even in samples not containing any TNF. We did not detect any HRP activity in any of the buffers or solutions used in the assay. However, we did find during these experiments that the streptavidin avidin/HRP concentration could be decreased from the initial 1:1000 dilution to 1:5000 concentration.

Microbial contamination proved to be a source of random result values. It was determined that the washing steps within the ELISA assay are essential for highest quality results. The wash buffer was initially prepared in mass volume quantity and stored at room temperature in the plate washer container and was not prepared fresh for each assay. Microbial growth slowly accumulated, and contamination was subsequently introduced during plate washing. It was determined that wash buffer must be prepared fresh per assay and stored at 4 °C for the duration of the assay. It was also determined that all tubing and containers of the automated plate washer must be pre-washed prior to every use in order to reduce as much contamination as possible.

Once the microbial contamination and incidental peroxidase activity were minimized our attention was turned towards to the assay reagents. The sandwich ELISA assay is a sequential assay that allowed for us to work in reverse order of addition of reagents to determine the
problematic constituent. This process lead to evaluating the assay diluent that is used within the ELISA assay as the blocking agent as well as the diluent of the secondary antibody and streptavidin/HRP. In the ELISA assay the well is initially coated with capture (1° antibody) and following that procedural step, the unbound capture antibody is removed and blocking reagent is added to the well to allow inert proteins to adhere to any unbound places within the well. The blocking reagent contains proteins from animal serum as well as the preservative ProClin-150. It was our hypothesis that over time the protein in the provided assay diluent began to break down; therefore, additional protein in the form of BSA was added to the diluent prior to each assay. The diluent was also aliquoted from the original bottle to 15 mL Falcon tubes to eliminate protein denaturation from several cycles of warming and cooling. These modifications were done to ensure more effective blocking of the wells.

When examining the possible sources of assay interference, we collected kinetic readings instead of the manufacturer’s suggested stopped assay readings. Kinetic collection allowed for a more accurate look at what was occurring over time after the addition of substrate to streptavidin/HRP. A set of criteria for data quality was created based on the initial rate of the colormetric change over time for each well. By examining the kinetic data we determined that the slope of color development over the first three minutes gave a good indication of whether the well would give a randomly high absorbance reading.

Application of our initial slope criterion is illustrated in Figure 3.1, which shows the results from cells with no added LPS to system. Each kinetic output represents an individual well of plated cells. As one can see, there are two samples that follow the same pattern by reaching a maximum absorbance of about 0.3 absorbance units (652 nm), while one sample reaches a maximum absorbance of about 1.6 absorbance units. The kinetic observation shows
that there is a gradual increase of absorbance over time for two of the three replicates illustrated, but a much more rapid increase in absorbance for the third replicate. Repeated experiments suggested to us that some wells on any ELISA plate that we prepared gave rapid initial absorbance changes and ultimately very high final absorbance values, presumably because of poor blocking and nonspecific sorption of the HRP in the last step of the assay. We used kinetic data like these to develop a stringent method for exclusion of wells that are apparently producing false readings. We determined the initial slope for each well over the first 3 min of the assay. If the initial slope was greater than 0.02 absorbance units/min, we did not use data from that well. For the data illustrated in Figure 3.1, initial slopes were 0.006, 0.049, and 0.005 absorbance units/min for replicates 1, 2, and 3, respectively. By our exclusion criterion, we would not include replicate 2 in our data set. Any well data that gave slope values that were higher than 0.02 absorbance units per min were discarded.

Although we collected kinetic data, we used the final absorbance value (60 min) for quantitation of TNF-α. We found that using the final absorbance value, or using the initial slope of the color development reaction, yielded very similar standard curves. It was easier and faster to record and analyze the final data point than to collect the initial rates data, and these data were also consistent with typical ELISA practice of using a stopped assay with fixed time of 1 h. TNF-α quantity was calculated by taking the final absorbance value of the accepted data wells and inserting into the equation given by the TNF-α standard curve (Figure 3.2).

The combined standard curve shown in Figure 3.2 represents the TNF-α standard absorbance readings from each assay. This combined standard curve allowed us to calculate the amount of TNF-α being produced under each set of variables. In future work we plan to expand the curve by assaying lower concentrations of TNF-α standards, which will allow a lower
detection limit. Figure 3.3 represents the absorbance values (652 nm) as a function of time for TNF-α standard concentrations from 0 pg/mL to 5000 pg/mL over a one hour time interval. This shows that the colormetric assay is working in a concentration-dependent fashion such that both the rate of formation of the colored species and the final intensity of color is larger as TNF-α concentration is increased.

Cells were isolated and verified by cell counting and viability by the absence of trypan blue visible within the cell as reported by Jeffers (12). Cells were allowed to adhere to the plates, then primed overnight with LPS (lipopolysaccaride) to mimic bactericidal stimulation and compared to untreated cells in which a low presence of TNF-α production was expected and observed (Figure 3.4). LPS-stimulated cells produced 1.4 times more TNF-α than unstimulated cells or blank wells.

Kolodzeij et al. (2001) (13) used human monocytes and measured TNF-α production in the presence of two polyphenols, castalagin and PGG, but in the absence of stimulants such as LPS. They noted that TNF-α was not produced at all by cells treated with PGG, and a low level was produced with castalagin stimulation. We tested the same tannins with our cell system at various concentrations to examine the hypothesis of tannins serving as an anti-inflammatory agent and suppressing inflammation.

For cells treated with no LPS, with LPS, or with PGG added simultaneously with LPS, we calculated TNF concentrations using the standard curve (Figure 3.5). In all samples treated with PGG, TNF-α production was elevated by the treatment. However, large errors made it impossible to establish significance. There was no obvious dose-response relationship.
Castalagin gave similar results, but lack the of purified tannin made it impossible to do sufficient replicates to obtain convincing data.

After doing several similar experiments, we normalized data by assigning a value of 1 to the amount of TNF produced by cells stimulated by LPS with no added phenolic. We plotted the normalized data and fitted them to a hyperbolic function to obtain an IC$_{50}$ value of 0.1 mg/mL PGG (Figure 3.6). The data suggest that if PGG has an effect on TNF-α production, it is a relatively small stimulatory effect and is saturated at a low concentration of PGG. Additional experiments with lower concentrations of PGG, to reach the dose-effect regions of the curve, are necessary to confirm this theory.

All in all, the data we obtained give us a foundation to understand the inflammatory response to the addition of tannins. We found that either PGG or castalagin, added in conjunction with LPS, increased TNF-α production in the monocytes. In contrast, Kolodjeiz reported that the addition of PGG and castalagin to his system induced little to no TNF. In his experimental system, the tannins were added to cells that were not stimulated with LPS, because his main interest was in developing effective inflammatory agents (13). Our data differ because our goal was to evaluate how tannins serve to suppress inflammation, by measuring suppression of TNF-α production that has been induced by the bacteria LPS. We are searching for tannins that are anti-inflammatory agents. Neither PGG nor castalagin appears to be a good anti-inflammatory agent, but we now have a system suitable for testing other tannins and protein-tannin complexes.

It is our hope that by switching to a more controlled CO$_2$ environment, via a CO$_2$ incubator, that our cell system will produce enhanced cells and induce more TNF-α, yielding a better system overall. In spite of various troubleshooting attempts, the ELISA assay still has
several difficulties that need to be corrected in order to make this assay a more reliable method for TNF-α quantitation. More optimization experiments have been planned in an effort to resolve the issues associated with the assay.
Figure 3.1  *Erratic replication.* ELISA assay of supernatants from cells with no added LPS. Each curve represents the kinetic output representing an individual well of plated cells. Two samples that follow the same pattern by reaching a maximum absorbance of about 0.3 absorbance units (652 nm), while one sample reaches a maximum absorbance of about 1.6 absorbance units. Exclusion method applied and replicate 2 was discarded from data set due to slope greater than 0.02 absorbance units/min. (ELISA_with_graphs: Media_3.08.07_ah_aeh)
**Figure 3.2 Combined standard curve.** Combined standard curve represents the TNF-α standard absorbance readings from each assay. (ELISA with graphs: 3.29.07)

\[ y = 0.543\ln(x) - 2.940 \]

\[ R^2 = 0.722 \]
Figure 3.3 TNF-α standard kinetic readings. Kinetic absorbance values (652 nm) yielded a dose response of TNF-α standard concentrations from 0 pg/mL to 5000 pg/mL over a one hour time interval. (ELISA with graphs 4_4_07: TNF Std 2.21.07)
Figure 3.4  Basal levels of TNF-α production. Supernatants from wells containing cells stimulated with LPS were compared to supernatants from unstimulated cells, media only, or empty wells  (ELISA with graphs: 3.08.07_aeh_aeh summary 2_21)
**Figure 3.5** TNF-α production by cells stimulated by LPS in the presence of PGG. Data presented for treatment with 0.25 mg/mL of PGG was not done in triplicate. (ELISA with graphs: 3.08.07_aeh_aeh summary 2_21)
Figure 3.6 TNF-α production in cells treated with various amounts of PGG. Normalized data were plotted and fitted it to a hyperbolic function and obtained an IC₅₀ value of 0.1 mg/mL PGG. (GraphPad: TNFgraphs.p3f-summary 3_07_07)
3.4. Chapter 3 References


Appendix 1.

FREE RADICAL SCAVENGING AND PROTEIN PRECIPITATION PROPERTIES OF GRAPE SEED EXTRACT

Andrei Prida¹, Akeysha A. Perkins², and Ann E. Hagerman²

Moldova Institute of Technology, Chisinau, MD-2064, Republic of Moldova
Department of Chemistry & Biochemistry, Miami University, Oxford OH 45056
hagermae@muohio.edu

Abstract

We investigated the radical scavenging activity of four fractions of grape seed extract using in vitro assays for superoxide radical scavenging, hydroxyl radical scavenging, peroxyl radical scavenging, and ABTS radical cation scavenging. The grape seed fractions were the crude water/alcohol extract, the fractions of the crude extract that were water soluble or alcohol soluble, and a fraction that was purified by sorption to polystyrene. Each fraction had a different capacity to scavenge radicals, with the crude extract and water soluble fraction much more effective scavengers of superoxide and hydroxyl radical than the other fractions. There was a strong correlation between ability to scavenge superoxide and hydroxyl radicals. The ability to scavenge peroxyl radicals was correlated to the ability to scavenge ABTS⁺. We found that the

¹ Moldova Institute of Technology
² Miami University  hagermae@muohio.edu
alcohol soluble fraction of the crude extract, and the polystyrene-purified fraction, were able to recycle iron but none of the fractions served as iron chelators to promote the Fenton reaction. The crude extract and water soluble fraction had highest tannin content based on protein precipitability measures, suggesting that tannins are more effective superoxide and hydroxyl radical scavengers than are nontannin phenols.

**Key Words.** Radical scavenger, antioxidant, tannin, polyphenolic, grape seed, protein precipitation.

**Introduction**

There is a currently much interest in the hypothesis that oxidative damage to biological molecules may be involved in the etiology of a wide range of diseases, including cancer and cardiovascular disease. Oxidative damage is the consequence of uncontrolled production of oxygen radicals that damage biological molecules including lipids, proteins, carbohydrates and nucleic acids (Davies, 1994, Jacob and Burri, 1996). Polymeric polyphenols (tannins) are naturally occurring antioxidants (Hagerman et al., 1998; Robinson, Maxwell and Thorpe, 1997; Simonetti, Pietta and Testolin, 1997) which occur in grapes and grape-derived beverages, cocoa, tea, medicinal herbs, berries, apples and other fruits (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Epidemiological studies provide evidence that the “French paradox” and other correlations of diet with health are due in part to the antioxidant activities of wine polyphenolics (Cooper, Chopra and Thurnham, 2004).

Precipitation of protein is a characteristic activity of the polymeric polyphenols known as tannins. Although the radical scavenging activities of many polyphenols have been assessed, less attention has been paid to correlations between antioxidant capacity and protein binding activity.
(Serafini Bugianesi, Maiani, Valtuena, De Santis & Crozier, 2003). The formation of polymeric polyphenolic-protein complexes is the crucial first step after ingestion of tannins. Complexation may result in loss of radical scavenging activity (Riedl and Hagerman, 2001) or may yield long-lived bioactive molecules that are effective gastrointestinal antioxidants long after food ingestion (Chen and Hagerman, 2004).

In the study described here, we evaluated free radical scavenging as well as protein-binding properties of grape seed extract, because grape seed is generally reported as a good source for antioxidants and radical scavengers (Castillo et al., 2000, Fan and Lou, 2004). Grape seed is a source of catechin, epicatechin, epicatechin-gallate, as well as procyanidin oligomers and polymers (Geny Saucier, Bracco, Daviaud, & Glories, 2003). Our goal is to define the relative activities of crude extracts and partially purified fractions with the long term goal of creating new therapeutic forms and dietary supplements from these abundant raw materials.

Several different approaches to measuring antioxidant activity of tannins were pursued, since no single in vitro approach adequately mimics in vivo systems. In the present study we focused on radical species that may be encountered in biological systems: superoxide, hydroxyl and peroxyl radicals. We also evaluated the grape seed extract using the model radical cation, 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺). Superoxide is produced during the inflammatory response and may be an important contributor to inflammatory diseases such as inflammatory bowel disease (Kruidenier and Verspaget, 2002). Hydroxyl radical is both a product of superoxide dismutation and of metal ion-catalyzed Fenton reactions (Aruoma, Grootveld and Halliwell, 1987). Polyphenolic compounds can potentially scavenge hydroxyl radical directly or can participate in Fenton reactions either by chelating metal ions or by recycling metal ions to their reduced state(Long, Clement and Halliwell, 2000). Peroxyl radicals
are intermediates in the oxidation of unsaturated lipids and proteins, and may transfer damage to other macromolecules (Gieseg, Pearson and Firth, 2003). The ABTS radical cation, which is not encountered in vivo, is a convenient compound for evaluating radical scavenging activity free of interference (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). Tannin content of grape seed fractions was assessed by measuring protein precipitability (Hagerman and Butler, 1978).

**Materials and Methods**

**Chemicals and Reagents.**

Deoxyribose, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid, trichloroacetic acid, nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), reduced β-nicotinamide adenine dinucleotide (NADH), and 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Chemicals (St. Louis, MO). All other chemicals were reagent grade. The grape seed fractions were a gift from “OenoConsulting”, Chisinau, Republic of Moldova. These preparations were obtained by extracting grape seeds with water-ethanol solutions and then fractionating the extract. The material provided as “Standard” (ST) was the ethanol-water extract of the grape seeds. That extract was evaporated under reduced pressure to remove ethanol, filtered, and brought back up to the initial volume with water to yield the fraction “Water-Soluble” (WS). The insoluble material from that step was redissolved in ethanol to yield the fraction “Alcohol-Soluble” (AS). The fraction identified as “Pharmaceutical” (PH) was obtained by purifying the initial standard
extract on a polystyrene-based adsorbent column. The exact compositions of these fractions were unknown, but grape seed polyphenolics are flavonoid-based.

**Superoxide scavenging**

The grape seed fractions were prepared at a concentration of 300 µg/mL in methanol. The poor solubility of AS was overcome by dissolving it in aqueous methanol (50:50, v/v). In this assay, superoxide anion radicals are produced when PMS is reduced NADH, and the superoxide immediately reacts either with NBT to form a colored species, or reacts with the test scavenger (Van Noorden and Butcher, 1989; Zou, Lu and Wei, 2004). The reaction was started by adding 38 µM NADH to mixtures containing 50 µM NBT, 10 µM PMS and varying concentrations (0-20 µg/mL) of scavenger in 3.0 mL of 16 mM Tris-HCl buffer pH 8.0. The initial rate of formation of NBT was determined at 560 nm over the first 20 s of each reaction, and the % scavenging was calculated by comparing the rate of formation of NBT in samples with scavenger to the rate in samples without scavenger. Each concentration of each fraction was assayed three times. The mixture without sample was the control and mixture without addition of PMS was used as the blank. The IC50 values were determined by plotting % scavenging as a function of grape seed fraction concentration.

**Hydroxyl radical scavenging**

The deoxyribose method was used to determine the ability of an antioxidant to quench the hydroxyl radical (Aruoma, 1994; Hagerman et al., 1998; Halliwell, Gutteridge and Aruoma, 1987). In the assay, hydroxyl radical damage to ribose is assessed by spectrophotometric determination of thiobarbituric acid reacting species (TBARS) generated in situ from deoxyribose by hydroxyl radicals generated by an iron-EDTA Fenton reagent and hydrogen
peroxide. The test compound competes with the deoxyribose for hydroxyl radical, and the concentration dependence of the competition reaction can be used to calculate the IC$_{50}$ for the reaction between test species and the hydroxyl radical. To maintain hydroxyl radical generation, Fe$^{+3}$ is recycled to Fe$^{+2}$ by ascorbic acid.

Reactions were performed in 10 mM phosphate buffer, pH 7.4, containing 2.8 mM deoxyribose, 2.8 mM H$_2$O$_2$, 25 µM FeCl$_3$, 100 µM EDTA, and the test material. Reactions were started by adding ascorbic acid to achieve a final concentration of 100 µM. In another series of experiments we used similar protocols but omitted either EDTA or ascorbate. After allowing the reaction to proceed for 1 h, thiobarbituric acid and trichloroacetic acid were added and the mixtures were heated in a boiling water bath for 20 min. Samples were allowed to cool, and the chromophore was extracted into n-butanol before reading the absorbance at 532 nm. Absorbances were compared to control samples not containing test material.

All organic compounds are hydroxyl radical scavengers, so test compounds must be dissolved in water rather than in ethanol for the deoxyribose assay. Because the grape seed fractions were not completely soluble in distilled water, we centrifuged all test solutions before using them in the assay. We calculated the concentrations of the solutions used in the deoxyribose assay by comparing the UV absorbances (280 nm) of the aqueous solutions to the absorbances of solutions prepared in water-ethanol (50:50 v/v) solutions.

**Peroxyl radical scavenging.**

Peroxyl radical scavenging activity of fractions was assayed *in vitro* by monitoring radical-mediated oxidation of iodide to iodine (Sano, Yoshida, Degawa, Miyase, & Yoshino, 2003). In our assay, 0.7 ml of sample solution was added to 2 ml acetonitrile-phosphate buffer
(1:1), followed by addition of 100 µl of saturated KI solution. After preincubation of the mixture at 39°C for 2 m, radical-induced oxidation was started by the addition of 200 µL of 0.5M 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH). After 60 m of incubation at 39°C in the dark, the reaction vessel was chilled immediately in an ice bath to stop the radical production by AAPH. After 5 m in the ice bath the volume of the reaction mixture was adjusted to 30ml with water. The concentration of molecular iodine in the mixture was determined by potentiometric titration with 0.25 mM Na2S2O3. Titration of sodium thiosulfate solution was performed using multi-parameter analyzer Consort C833 equipped with a platinum combination electrode, model SP60X (Consort, Belgium) and electronic pipettes (Arise Biotech Co, Taiwan). The minimum titration amount of Na2S2O3 was fixed at 0.1 mL and the maximum potential difference was taken as the end point of the titration. The radical scavenging activity was determined by comparing the amount of iodine produced in the presence of fractions to the amount produced in the absence of any antioxidant. The concentration required to inhibit iodine production by 50%, or the IC50, was calculated for each fraction.

**ABTS radical scavenging**

The ABTS radical cation was prepared as described by Re et al. (1999). Typically, 15 mL of 7.01 mM ABTS (3.84 mg/mL) was prepared in 20 mM phosphate buffer, pH=7.4, and mixed with 1 mL of 39.2 mM K2S2O8 (10.6 mg/mL) dissolved in the same buffer. The mixture was incubated at room temperature in the dark overnight and was then diluted with buffer to obtain the desired working solution of radical cation. Radical concentration was determined spectrophotometrically at 734 nm based on the extinction coefficient (E280 = 12867 M⁻¹ cm⁻¹). Up to 100 µL of putative radical quenching agents dissolved in ethanol:water (1:1, v:v) was added to 1000 µL of 65 µM ABTS⁺. The absorbances of both control and samples were read
after 10 m of incubation. The ABTS$^{+}$ scavenging activity was calculated by comparing the absorbance of the test solution to that of the control. The concentration required to scavenge 50% of the ABTS$^{+}$ (IC$_{50}$) was calculated for each fraction.

**Protein precipitation assay**

Protein precipitable phenolics were determined using BSA (Hagerman and Butler, 1978). The grape seed preparations were dissolved in aqueous ethanol (50:50, v/v) and 200 µL of the sample was added to 250 µL of 0.2 M sodium acetate buffer (pH=4.9) containing 50 µg of BSA. After 30 m at room temperature, the precipitates were removed by centrifugation at 13000 x g, the supernatants were aspirated, and the precipitates were dissolved in 800 µL of SDS-TEA (sodium dodecylsulfate 5% (w/v), triethanolamine 1% (v/v)) solution. The solution was mixed with 200 µL of ferric chloride solution (0.01 M FeCl$_3$ in 0.01 M HCl) and after 15 m the absorbance was read at 510 nm.

**Results and Discussion.**

The ability of the grape seed fractions to scavenge four radicals was determined by calculating the amount of compound to inhibit radical formation by 50% (IC$_{50}$) (Table 1). Not surprisingly, we found that the different fractions of grape seed extracts reacted with different efficiencies for the four radicals tested. In general, the alcohol-soluble (AS) fraction was the least potent radical scavenger. We used simple correlation analysis to compare the results obtained with the four radicals. We found that superoxide radical scavenging was highly
correlated with hydroxyl radical scavenging \( (r^2 = 0.94) \) but not with peroxyl \( (r^2 = 0.46) \) or ABTS \( (r^2 = 0.46) \) radical scavenging. Similarly, hydroxyl radical scavenging was poorly correlated with either peroxyl radical \( (r^2 = 0.41) \) or ABTS \( (r^2 = 0.58) \) scavenging. However, ABTS radical and peroxyl radical scavenging results were correlated \( (r^2 = 0.76) \).

For simple radical scavengers, the deoxyribose assay can be used to estimate the rate of reaction of a scavenger with the hydroxyl radical. Our estimated rate constants for all four fractions \( (1-10 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}) \) are similar to values obtained for many other organic compounds, but interpretation of those rate constants is complicated by potential side reactions characteristic of polyphenolics (Hagerman et al., 1998, Lopes, Schulman and Hermes-Lima, 1999). The deoxyribose method relies on the reduction of hydrogen peroxide by EDTA-chelated \( \text{Fe}^{2+} \), and on ascorbic acid recycling the iron to maintain constant rates of hydroxyl radical production. Ortho-diphenols are metal ion chelators, and reduce iron. In addition to scavenging hydroxyl radical, polyphenolics may promote hydroxyl radical formation, and may thus have pro-oxidant activity in addition to antioxidant activity. These interfering reactions can be detected by running deoxyribose assays in the absence of either ascorbic acid or EDTA.

We found that omitting ascorbic acid from the reaction in the absence of polyphenolic suppresses 80% of the TBARS formation, because \( \text{Fe}^{2+} \) is depleted early in the reaction without iron recycling. Similar low levels of TBARS were formed when fractions ST and WS were substituted for ascorbate, but fractions AS or PH partially restored TBARS formation when substituted for ascorbate (Table 2). Omitting EDTA from the reaction in the absence of putative scavengers suppresses 90% of the TBARS formation, because only chelated iron can participate in the Fenton reaction. None of the grape seed fractions was an effective substitute for EDTA although PH may have some metal-chelating activity (Table 2). Our work confirms earlier
suggestions that the ortho-dihydroxyl and trihydroxyl phenolics found in grape seed are potentially both iron complexing and iron recycling agents (deFreitas, Glories and Laguerre, 1998).

The correlation we noted between ABTS+ and peroxyl radical scavenging activities suggests that the ABTS assay be adopted as a useful alternative to the technically much more difficult peroxyl radical scavenging method. The ABTS assay does not adequately predict superoxide or hydroxyl radical scavenging activity. The superoxide assay is simpler than the deoxyribose assay, and gives results that correlate well with hydroxyl radical scavenging. However, the deoxyribose assay is uniquely capable of providing information about the ability of some compounds to promote oxidative damage via Fenton pathways.

The protein precipitable phenolics assay was used to evaluate the amount of tannin in each fraction (Hagerman and Butler, 1978). Unlike simple phenolics, tannins react with protein to form cross linked, insoluble complexes. Tannin-rich extracts are multivalent for protein binding, and saturate protein at rather low concentrations (Hagerman, Rice and Ritchard, 1998) while extracts depleted in tannins are less efficient precipitating agents. For all four grape seed fractions, when small amounts of the fractions were added to the protein, precipitable phenolics increased linearly with the amount of extract added (Fig. 1a, b). For the crude extract ST and the water-soluble fraction WS, the protein was saturated with tannin when more than 1 mg of the fraction was added (Fig. 1a),. The alcohol-soluble (AS) and column purified (PH) fractions do not saturate the protein even when large excesses of the fraction are added (Fig. 1b), because these samples contain less tannin, and are less effective precipitating agents. Even when large excesses of these fractions have been added to protein, the protein is only partly precipitated, so
there is a linear relationship between extract added and precipitable phenolic at all levels of phenolic.

Comparing the radical scavenging activities to the tannin composition of the reveals that higher tannin content leads to more potent superoxide and hydroxyl scavenging activity. The crude extract, ST, and the water-soluble fraction SW, were tannin-rich and also were the most effective superoxide or hydroxyl radical scavengers. In addition, these tannin-enriched fractions did not have pro-oxidant activities in the hydroxyl radical scavenging assay. The grape seed fractions that contained more nontannin phenolics (AS, PH) were not as effective scavengers. Our observations were consistent with earlier reports of the efficacy of tannins in radical scavenging assays, compared to low molecular weight phenolics (Hagerman et al., 1998, Yokozawa, Chen, Dong, Tanaka, Nonaka, & Nishioka, 1998). These results suggest that the tannin-rich fractions of grape seed extracts should be pursued for human health benefits as natural antioxidants.

Acknowledgements.

The authors thank Moldavian Research and Development Association for financial support (grant MTFP-023/05 #TFP-12 to A.P.); NIDDK R15 DK069285 to A.E.H; and “OenoConsulting” S.R.L. for supplying us with grape seed extracts. Kim Wisman assisted with ABTS scavenging assays.
References.


Table 1. The results of different scavenging assays. Chemical systems for generating superoxide radical, hydroxyl radical, peroxyl radical or the ABTS radical cation were used to evaluate the scavenging activity of the grape seed fractions. Fractions were tested at several concentrations and the IC₅₀ calculated as the concentration to scavenge half of the radical under standard conditions. Tested fractions were crude extract (ST), water-soluble fraction (WS), alcohol-soluble fraction (AS) and column-purified fraction (PH). Values represent the mean of at least three determinations, with standard deviations.

<table>
<thead>
<tr>
<th>Grape seed fraction</th>
<th>Superoxide scavenging IC₅₀ µg/mL</th>
<th>Hydroxyl radical scavenging IC₅₀ mg/mL</th>
<th>Peroxyl radical scavenging IC₅₀ µg/mL</th>
<th>ABTS scavenging IC₅₀ µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST</td>
<td>3.42 ± 0.19</td>
<td>1.92 ± 0.27</td>
<td>39.4 ± 0.3</td>
<td>1.01 ± 0.04</td>
</tr>
<tr>
<td>WS</td>
<td>6.74 ± 0.56</td>
<td>1.71 ± 0.73</td>
<td>45.9 ± 0.4</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>AS</td>
<td>18.4 ± 1.7</td>
<td>8.71 ± 3.56</td>
<td>50.7 ± 0.6</td>
<td>1.49 ± 0.04</td>
</tr>
<tr>
<td>PH</td>
<td>8.51 ± 0.56</td>
<td>3.19 ± 0.22</td>
<td>36.2 ± 1.5</td>
<td>0.57 ± 0.05</td>
</tr>
</tbody>
</table>
Table 2. Pro-oxidant activity of polyphenolics detected with the deoxyribose assay. Pro-oxidant activity of polyphenolics detected with the deoxyribose assay. The deoxyribose assay was used to evaluate the ability of grape seed fractions to recycle iron (ascorbate omitted) or to chelate iron (EDTA omitted). Each fraction was tested at 2.9 mg/mL. Values shown are TBARS production as a % of the value obtained in control reactions containing both ascorbate and EDTA, and no grape seed fractions. Means of three determinations and standard deviations are given.

<table>
<thead>
<tr>
<th>Grape seed fraction</th>
<th>Ascorbate omitted</th>
<th>EDTA omitted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBARS as % of control (with ascorbate)</td>
<td>TBARS as % of control (with EDTA)</td>
</tr>
<tr>
<td>None</td>
<td>24.0 + 5.5</td>
<td>10.2 + 2.1</td>
</tr>
<tr>
<td>ST</td>
<td>21.2 + 4.3</td>
<td>3.1 + 0.5</td>
</tr>
<tr>
<td>WS</td>
<td>10.0 + 0.7</td>
<td>8.9 + 3.1</td>
</tr>
<tr>
<td>AS</td>
<td>37.3 + .9</td>
<td>8.9 + 0.2</td>
</tr>
<tr>
<td>PH</td>
<td>46.6 + 7.4</td>
<td>17.1 + 2.5</td>
</tr>
</tbody>
</table>
Figure 1. Protein (50 µg) was incubated with grape seed extract at pH 4.9 for 30 min before isolating the precipitate by centrifugation. Precipitates were redissolved in detergent and base, and phenolics in the precipitates were measured after formation of the purple iron-phenolate complex. The points represent the averages of two determinations that differed by less than 2%. The line indicated has a slope of 0.5 mg phenolic precipitated per mg extract added. (a) Crude extract ST, water-soluble fraction WS.
Figure 1. (b) Alcohol soluble fraction AS, column-purified fraction PH.
Appendix 2. Composition of Cottonwood Tannins

Appendix 2.1. Introduction

Chemical composition of plants is essential in understanding the plants diversity, which can be a function of genetic, developmental and environmental influences within species (1). Chemical composition includes both primary nutrients such as protein and lipids, and secondary compounds such as polyphenolics and alkaloids. Cottonwood contains several phenolics including condensed tannins. Condensed tannins, or proanthocyanidins, are polymers of two or more flavonoid units, conjoined by carbon-carbon bonds, not allowing for hydrolysis to occur. Condensed tannins can be degraded to pigments known as anthocyanidins. For cottonwood tannin, genetics and development have been deemed more important than seasonal changes based on measures of total tannin content by Rehill et al. (1). To evaluate this further we examined the detailed chemical composition of cottonwood species and their hybrids.

Condensed tannins found in the cottonwood species Fremont (*Populus fremontii*), narrowleaf (*P. angustifolia*), and their F1 hybrids were assessed. Fremont cottonwood is usually found at lower elevations while narrowleaf is found at higher elevations. The F1 hybrid occurs naturally as these two elevations merge and the plants distribution overlaps. We hypothesized that genetic diversity in plants, such as the cottonwood, would be reflected in its chemical diversity via secondary chemicals such as tannins.

Appendix 2.2. Methods

The general strategy for compositional analysis of condensed tannins is degradation of the polymer in acidic solution in the presence of a nucleophilic thiol such as toluene $\alpha$-thiol. Extender units are trapped as the thiol adducts, and can be separated and quantitated by HPLC
(high performance liquid chromatography). The terminal unit is released as the free flavan-3-ol. In order to determine the tannin composition of our samples, tannin standards were first purified from natural sources identified from the literature. Sorghum, willow, and sainfoin tannins were reacted with the thiol to yield standards for epicatechin, catechin, and epigallocatechin thiols respectively.

The products of thiolysis were chromatographed with Sephadex LH-20. The column elutes terminal units, thiol adducts, and unreacted thiolysis reagents. The elute is monitored spectrophotometrically at 280 nm, and fractions are evaporated down, and run on HPLC to evaluate purity. After tentative identification by HPLC, electrospray mass spec was performed for final identification of the individual pure thiols.

Cottonwood samples provided by Rick Lindroth (University of Wisconsin) were treated via thiolysis reaction and run on HPLC. The thiolysis reaction shown below (Figure Appendix 2.1) was carried out by adding 25 μL of acidic methanol in a micro-centrifuge tube along with a tip of a spatula amount of the cottonwood sample. 12 μL of 5.0 % toluene-α-thiol (TαT) was added to the sample and mixed by vortexing. The mixture was incubated at 40° C for 30 minutes and was immediately transferred to the freezer for 5 minutes in order to stop the reaction. The sample was brought up to a total volume of 100 μL with the addition of acidic methanol, and filtered using a 0.45 μm nylon membrane non-sterile micro-spin filter. A C-18 column was used for HPLC separation starting with a 100% of mobile phase A (low organic phase) and 0% of mobile phase B (high organic phase) Mobile phase B was slowly increased over the 50 minute run while mobile phase A was decreased respectively. Mobile phase A is prepared by dissolving 4.14g of monobasic sodium phosphate in 800 mL of nanopure water. The pH of the solution was then adjusted to 3.0 using NaOH. 10 mL of MeOH was added to the mixture along
with 100 µL of trifluoroacetic acid (TFA). The solution was then brought to a final volume of 1L using nanopure water to yield a final concentration of 0.01% TFA. Mobile phase B was prepared in the same fashion with the addition of 600 mL of MeCN. Peaks were compared to standards in order to identify composition.

**Appendix 2.3. Results and Discussion**

In this preliminary study the two parent species, Fremont and narrowleaf, had distinctly different compositions and chain lengths (Figure Appendix 2.4). The F1 hybrid was intermediate between the parents but was more similar to the Fremont. This is similar to the results obtained in the earlier study which examine quantities of tannin accumulated in the plants. Quantitatively Fremont had very little tannin, narrowleaf was tannin-rich, and the F1 hybrids had intermediate, but relatively small, amounts of tannin. Thus the compositional differences that we noted mirrored the quantitative differences found in an earlier study. However, we analyzed samples from only a few individual plants of each type. We do not yet know whether individual variation is a significant contributor to the compositional differences we noted in cottonwood tannins.
**Figure Appendix 2.1.** *Thiolysis reaction.* The thiolysis reaction illustrated with a condensed tannin dimer comprised of epicatechin extender and catechin terminal unit.
**Figure Appendix 2.2. HPLC standards.** Typical chromatograms showing the thiol adducts of epigallocatechin (EGC), catechin, and epicatechin. The second chromatogram shows elution times for the terminal units, released as the free flavan-3-ols catechin or epicatechin.
Figure Appendix 2.3. Chromatogram of representative narrowleaf cottonwood sample after thiolysis reaction. This plant tannin had terminal catechin units. Extenders were predominantly epigallocatechin with relatively little epicatechin.
Figure Appendix 2.4. *Relative moles of condensed tannin extender units and chain length.* Peak areas are converted to moles of released terminal and extender units to calculate relative moles of extender and average chain length for the tannin in samples from a given species.
2.4. Appendix 2 References