ANTIOXIDANT MECHANISMS AND BIOACTIVITY OF PHENOLIC COMPOUNDS FOUND IN *DIOSCOREA BULBIFERA*

by

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ABSTRACT

ANTIOXIDANT MECHANISMS AND BIOACTIVITY OF PHENOLIC COMPOUNDS FOUND IN *DIOSCOREA BULBIFERA*

Patricia Elizabeth Rees

Accumulation of oxidative damage has been implicated in numerous diseases bringing antioxidant investigations and plant phenolics to the forefront of medicinal research. The species *Dioscorea bulbifera* has been used in both traditional and modern medicinal systems and has good overall antioxidant capacity. Yet little work has been done to assess which chemical components may contribute to this action. The goals of this study were to investigate the antioxidant activity of individual phenolic compounds in the plant *D. bulbifera*; to compare the in vitro antioxidant assays with human cell studies; for this information to contribute to future investigations in treating human disease.

Methanolic extracts of the D. bulbifera bulbils were analyzed using HPLC-MS/MS and NMR. Positively identified compounds were used for in vitro antioxidant assays (Ferric Reducing Antioxidant Power (FRAP), iron chelation, and Oxygen Radical Absorbance Capacity (ORAC), followed by human cell assays.

Several phenolic species were tentatively identified. (+)-catechin was positively identified and used for further testing. In vitro assays showed potential for electron transfer antioxidant activity but not iron chelation. The hydrogen transfer mechanisms could not be assessed due to assay complications. Cell assays also suffered complications rendering them inconclusive.

CHAPTER 1
INTRODUCTION

In recent years, it has become commonplace to see commercials or advertisements advocating the consumption of antioxidants as part of a healthy lifestyle. Foods with the highest concentrations of antioxidants are termed superfoods and marketed as miracle compounds able to slow aging and prevent illness (Gutteridge and Halliwell, 2010). Antioxidants are also consistently added to foods, beverages and cosmetics to inhibit oxidation and spoilage (Shahidi and Ambigaipalan, 2015). With such widespread use, one might assume that these claims are well studied and supported. However, within the scientific community there is little agreement regarding the efficacy, safety, and outcome of antioxidants in foods, health and beauty supplements, or medicines. Truly, little is known about which antioxidants are beneficial, how much is safe to consume, or how they react within the body. Many review articles on the subject clearly express a dire need for standardization of testing, better chemical characterization of compounds, and the use of testing with more biological relevance (Gutteridge and Halliwell 2010; Niki 2010; Negi 2012; Bast, Haenen and Guido 2013; Comert and Gokmen 2018).

The trend of increased antioxidant usage was simultaneously fueled by the desire to improve human health and the increasing skepticism regarding the use of synthetically produced compounds (Bast, Haenen and Guido 2013). Numerous studies have noted that oxidative imbalance is a common factor among diseases such as cancer, arteriosclerosis, and diabetes. Others have noted that exogenous antioxidants can help to minimize or reduce the chances of these diseases (Pisoschi and Pop 2015). These findings in conjunction with the public's desire for more "natural" additives in food and beauty products propagated a growing interest in antioxidant compounds from plants. Of the many classes of phytochemicals, phenolic compounds have been shown to be particularly useful antioxidants in vitro (Comert and Gokmen

2018). Unfortunately, once the potential health benefits of antioxidants, including phenolic compounds, were brought to light many misconceptions about their use began to flourish. Of particular concern are the beliefs that all antioxidants are beneficial, naturally sourced means safe, and more is always better (Bast, Haenen and Guido 2013).

Recently, the more negative aspects of these compounds have become evident. It is now known that antioxidants can be both beneficial and deleterious depending on their dosage and environment (Gutteridge and Halliwell 2010). Based on these findings, the exploration of antioxidant compounds must be expanded to address current knowledge gaps regarding antioxidant function and biological interaction. One concern is that past studies relied heavily on *in vitro* rather than *in vivo* assays. By design these studies have questionable physiological implications because they fail to address the complex reactions that would take place in a living organism. Additionally, little emphasis was placed on testing individual compounds or their mechanism of action. As such only vague conclusions could be drawn about how antioxidants "may" be beneficial in preventing disease. Currently research is needed to fill in these gaps so that we can make more informed decisions about antioxidants in medicine, food, and cosmetics (Martins et al 2016).

This study contributes to past research by narrowing the focus from overall phenolic activity to the specific mechanisms of individual plant phenolic compounds. Multiple in vitro assays were performed to elucidate the possible chemical mechanisms of individual compounds, rather than general antioxidant potential of a whole extract. These tests were followed by human cell-based assays to analyze the bioactivity and potential cytotoxicity of the compounds in a living system with the overall goal of improving the way we study antioxidant compounds.

Oxidative Reactions in a Healthy System

Under ideal circumstances reductive and oxidative reactions are maintained in a strict balance with both playing a vital role in proper physiological function. Oxidative species include a wide range of compounds such as free radicals, reactive oxygen species (ROS), and reactive nitrogen species. The focus of this study is reactive oxygen species. Common examples are superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and nitric oxide ($NO^{\bullet}$) radicals (Phaniendra et al 2015). These play critical roles in electron transfer chains used for chemical synthesis within cells, aid in pathogen defense, and act as signaling molecules during apoptosis and other biological processes (Gutteridge and Halliwell 2010; Rajendran et al 2014; Pisoschi and Pop 2015) . Antioxidants include both exogenous compounds absorbed from the foods we eat as well as endogenous compounds and enzymes produced within the body. Exogenous sources include vitamins C and E (Rajendran et al 2014). Phytochemicals like the resveratrol found in red wine, and catechins in green tea are also part of this group (Oroian and Escriche 2015). Endogenous antioxidants include catalase, superoxide dismutase and glutathione (López-Alarcón amd Denicola 2013). All antioxidants react with oxidative species to neutralize them, or make them less reactive so they do not damage nearby cells or tissues (Rajendran et al 2014).

An example of the usefulness of ROS is the pathogen response seen in the human immune system involving hypochlorous acid (HOCL). When certain pathogens are detected, dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase begins to produce superoxide that dismutates into hydrogen peroxide and is converted into HOCL by myeloperoxidases inside of neutrophils. Much like the bleach in household cleaning products, the HOCL produced by cells kills pathogens before they can cause serious infection (Knoefler et al 2014). Under the current ideology about ROS compounds, NADPH oxidase, superoxide's,

peroxides and HOCL are agents of disease and must be eliminated. The more logical conclusion is that without oxidative reactions such as this one, many valuable defense systems would be rendered ineffective. Another example of a beneficial ROS species is $NO^{\bullet}$. Under normal conditions $NO^{\bullet}$ plays a vital role in the regulation of blood pressure by promoting relaxation of the smooth muscles within blood vessels. It is also helpful in maintaining normal platelet aggregation and balancing smooth muscle growth and differentiation (Phaniendra et al 2015; Bryan 2018). Again, this highlights the misconception that all ROS are harmful to human health.

Unfortunately, there are situations that disrupt the oxidative balance. Increases in poor diet, pollution, disease, and exposure to some chemicals or drugs all increase the bodies oxidative load and can overwhelm natural antioxidant mechanisms. As the concentrations of oxidative compounds rise, these compounds react with nearby cells, proteins, and genetic material degrading them at a chemical level (Prior 2015). Over time this can lead to an accumulation of oxidative damage which may contribute to diseases such as rheumatoid arthritis (Veselinovic et al 2014), osteoarthritis (Mobasheri and Batt 2016), Alzheimer's (Poprac et al 2017), and atherosclerosis (Li et al 2014). Likewise, certain diseases such as cancer and diabetes are marked by abnormal metabolic function that results in increased oxidative stress which can exacerbate the damaging symptoms of the disease (Poprac et al 2017; Sarangarajan et al 2017). As a result, methods for controlling oxidative imbalances in the body have become a focus for maintaining human health. Regrettably, this focus tends to be very narrowly defined, with ROS species being villainized and antioxidants being glorified for their disease preventing properties.

When Antioxidants Misbehave

Broad claims about the risks of ROS and the benefits of antioxidants are risky. Based on such assumptions, it has become common place to add antioxidant compounds to foods to preserve freshness by minimizing oxidation of fats and oils (Jiang and Xiong 2016; Carocho et al 2018). However, not all antioxidant compounds are safe to consume. Even common antioxidants found in foods such as vitamin E can be potentially dangerous under the right circumstances (Vrolijk et al 2015). To make matters worse, some antioxidants may act as pro-oxidants in certain parts of the body, under certain conditions (Gutteridge and Halliwell 2010).

For years, the synthetic antioxidant compounds, butylated hydroxytoluene and butylated hydroxyanisole have been added to foods as preservatives. Recently, however, these compounds were shown to promote liver damage and were identified as being potentially carcinogenic (Shahidi and Ambigaipalan 2015). Now, they are slowly being replaced with compounds such as vitamin C and E which were believed to be safer and healthier. However, more extensive studies show that the benefits of these compounds may vary. For instance, vitamin E has been shown to increase the carcinogenic activity of dimethylbenz-(a)anthracene found in cigarette smoke and therefore may pose negative risks for habitual smokers (Vrolijk et al 2015).

Another potentially harmful outcome of indiscriminately increasing antioxidant intake is that antioxidants do not reduce the level of oxidative species in every environment. Under certain circumstances antioxidants can act as pro-oxidants, increasing free radicals and ROS in the body (Amorati and Valgimigli 2015). An example of this behavior is seen in the interaction of some antioxidants with metal ions. In particular, Fe^{3+} and Cu^{2+} have a propensity for reduction by antioxidants. In their reduced state, these metal ions react with compounds such as hydrogen peroxide producing hydroxyl and/or alkoxyl radicals. The resulting radicals initiate the same

oxidative chain reactions that antioxidants are supposed to prevent (Amorati and Valgimigli 2015).

Antioxidant Classification

Antioxidants are known to act in several ways to minimize oxidative damage and are typically classified by how they interfere with oxidative chain reactions. Under ideal conditions an antioxidant (AH) might interact with a free radical ($FR^{\cdot}$) to neutralize it before interacts with any other compounds. However, in some circumstances a secondary free radical ($A^{\cdot}$) is formed that can then react with nearby biological molecules (BTH) such as fats or proteins radicalizing them ($BT^{\cdot}$). This sets off a chain reaction wherein $A^{\cdot}$ is free to react with oxygen, forming a peroxyl radical ($AOO^{\cdot}$), or a superoxide ($O_2^{\cdot -}$). Superoxides can then be broken down by superoxide dismutase into hydrogen peroxide (H_2O_2) which has the potential to generate new hydroxyl radicals in the presence of certain metal ions, restarting the chain reaction. Cumulatively such reactions can cause major oxidative damage to tissues in a relatively short amount of time if uninhibited (López-Alarcón and Denicola 2013). The general sequence of the reaction is as follows:

$AH + FR^{\cdot} \rightarrow A^{\cdot} + FRH$ (1)
$A^{\cdot} + BTH \rightarrow AH + BT^{\cdot}$ (2)
$A^{\cdot} + O_2 \rightarrow AOO^{\cdot}$ (3)
$A^{\cdot} + O_2 \rightarrow A_{OX} + O_2^{\cdot -}$ (4)

Because of this complex reaction cycle, antioxidants are often classified as either preventative, competitive, or indirect, depending on where they interfere in the chain reaction. Preventative antioxidants inhibit initiation through chelation of metal ions, preventing them from interacting with H_2O_2. They may also directly neutralize an oxidative species before any reaction with the substrate can occur (inhibition of equation 1). Endogenous antioxidants such as catalase, which converts H_2O_2 to water and oxygen, often fulfill this role (Amorati and

Valgimigli 2015). Competitive antioxidants, also known as “chain breakers” are compounds that react with secondary peroxyl radicals (equation 3) to stop or slow their reaction with the substrate. An antioxidant may do this through either electron transfer, or more commonly, by hydrogen atom transfer. Indirect antioxidants act by stimulating the expression of endogenous antioxidants, rather than through direct reaction with ROS (Amorati and Valgimigli 2015).

Plant Phenolic Compounds and Prevention of Oxidative Imbalance

As interest in antioxidants has grown, many researchers have turned to plant secondary metabolites as a potential source. This may be partially due to the public misconception that ‘natural’ food additives are inherently more safe (Bast, Haenen and Guido 2013). The moniker ‘secondary plant metabolites’ can be attributed to the fact that they do not contribute directly to primary metabolic processes such as glycolysis or the tricarboxylic acid cycle. Rather, they serve equally critical roles in pathogen defense, molecular signaling, structural support, and prevention of oxidative damage. Phenolic compounds account for at least 8,000 of these secondary metabolites and many studies have supported their antioxidant activity. For this reason, many believe they may provide a means for prevention of oxidative damage and related diseases in humans (Shahidi and Ambigaipalan 2015; Zhang and Tsao 2016).

Dioscorea bulbifera

Dioscorea bulbifera is an herbaceous vining plant originating from Africa. This species has large, dark green, heart shaped leaves but is best known for its bulbils. The dense potato like fruits with scaly brown skin are produced on the vines giving the plant its common name, “air potato” (Langeland, Enloe, 2001). Although less common in the United States, many species of the genus *Dioscorea* are cultivated world-wide for food and medicinal uses. Traditional uses include treatment of sores, skin disorders, tumors, ulcers, and inflammation (Kumar et al 2013).

In modern medicine, *Dioscorea* spp. have been used as a source of phyto-estrogen precursors for oral contraceptives and diosgenin, a cancer treatment compound (Kumar et al 2013; Ghosh et al 2014). Long term medicinal use by multiple communities makes this genus a good candidate for continued research. Since *D. bulbifera* grows in the southeastern United States it is more readily accessible than other species of this genus making it a reasonable focus for this study.

The Role of Neutrophils in Human Health and Disease

Neutrophils are a very abundant type of leukocyte from the myeloid lineage. They are produced from hematopoietic stem cells in the bone marrow in a process referred to as granulopoiesis due to the distinct granules that are present in the resulting cells (Wang and Arase 2014). Neutrophils are the most prominent leukocytes found in the blood and their rapid response makes them indispensable during innate immune responses (Cascão et al 2010). The primary mode of pathogen defense deployed by neutrophils is phagocytosis. During this process pathogens, or their by-products, are engulfed by the neutrophil and sequestered into vesicles. Once inside, pathogens are exposed to a variety of enzymes, antimicrobial compounds and ROS from the granules. This process ultimately kills both the neutrophil and any pathogens contained inside it within minutes (Pruchniak et al 2013)

In addition to phagocytosis, neutrophils undergo respiratory burst, a series of reactions that result in the conversion of oxygen into ROS. For example, the NADPH oxidase system converts molecular oxygen into more reactive compounds such as superoxide ions (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2). These compounds are not usually reactive enough to degrade pathogens alone, but they encourage the formation of more reactive radicals that perform this function (Pruchniak et al 2013). Another critical enzyme held in the granules is myeloperoxidase (MPO). During phagocytosis, MPO catalyzes the production of

hypochlorous acid (HOCL), reactive nitrogen species and various protein radicals to help neutralize pathogens (Odobasic et al 2016).

An alternative to phagocytosis is the production of nuclear extracellular traps (NETs). Upon stimulation by chemical mediators or bacterial stimuli, the neutrophil extrudes a dense mass of chromatin, histones, and enzymes (Pinegin et al 2015). The resulting web traps pathogenic cells and debris, preventing them from spreading. Research also suggests that granule contents like MPO and HOCL in NETs may assist in killing the trapped pathogens (Odobasic et al 2016).

Neutrophil recruitment and function are tightly controlled processes, regulated by a variety of signals throughout the body. The complexity of this system creates many opportunities for neutrophils to become dysregulated, possibly contributing to disease. The reactive nature of their granule components is a significant cause for concern. The contents are specifically produced to cause maximum damage to proteins, DNA, and lipids to kill pathogens. However, their reaction with human cells can be just as destructive (Soehnlein et al 2017). In patients with rheumatoid arthritis, the ROS and other enzymes released by neutrophils can catalyze reactions that degrade collagen and other joint components. For instance, the excretion of hypochlorite increases the ability of collagenase to break down collagen in the affected joints worsening disease symptoms and damage (Fattori et al 2016). Because of their potential destructive capacity, neutrophils are a good starting point for human cell-based antioxidant research.

Improved Methods and Ideology for Better Human Health

Clearly the roles of antioxidants and ROS in the human body are not as simple as researchers initially thought. Many studies have indicated that over accumulation of ROS may lead to degenerative disease. Still, we must be more selective about how we use antioxidants to

avoid overconsumption or the use of potentially harmful compounds in foods and cosmetics (Veselinovic et al 2014; Mobasheri and Batt 2016; Poprac et al 2017; Sarangarajan et al 2017). Accomplishing this task requires that researchers better understand the chemical mechanisms of antioxidant compounds and their biological interactions (Gutteridge and Halliwell 2010; Comert and Gokmen 2018). To support this need, some of the phenolic compounds isolated from the *Dioscorea* samples were selected for antioxidant testing. The antioxidant assays focused on the preventative antioxidant activity by metal chelation of iron ions since this is a common starting point for many antioxidant chain reactions. Investigation of the chain breaking activity included assays for both electron transfer and hydrogen atom transfer mechanisms. Indirect antioxidant activity was not be included in this study.

In vitro tests alone cannot always predict how a compound will react within a biological system. Such tests are carried out under conditions that may not exist in the body and are not inhibited by structures such as cell membranes and endogenous enzymes that govern the reactions *in vivo* (Bender and Graziano 2015). While live animal or human testing is the ultimate method to resolve this issue, it is not only expensive, but risky until a compound has been well characterized. Before considering live trials, cell-based assays are often employed. Therefore, the chemical assays in this study were followed by antioxidant and cytotoxicity assays investigating the interaction with human cells. The antioxidant capacity of the sample compounds is based on the ability to penetrate the cell membrane and decrease the oxidation of a fluorescent probe within the cytoplasm (see the methods section for more detail).

Cytotoxicity was assessed by evaluating at the level of apoptosis and necrosis induced by different concentrations of the sample compounds. One of two outcomes was expected. First, the sample compound could protect the cells reducing the level of apoptosis when compared to a

positive control. Conversely, the compound itself could be cytotoxic and promote apoptosis (de Blas et al 2016). While cellular protection is an important factor when looking for potential medicinal compounds, being cytotoxic does not mean a compound is useless. Compounds determined to be cytotoxic at low levels may be harmful to healthy cells, so they may not represent good broad-spectrum treatments. However, such compounds have been shown to be useful in targeting cancer cells and therefore may still have medicinal potential (Khonkarn et al 2010; de Blas et al 2016).

Hypotheses

Hypothesis 1

It is expected that the selected compounds will be minimally toxic to human cells at low concentrations. The null hypothesis is that the selected compounds are cytotoxic at low concentrations. Cytotoxicity was assessed by monitoring a fluorescent dye which only stains dead/necrotic cells.

Hypothesis 2

Fluorescence of a dye which accumulates in the cytoplasm of the cell and is colorless until oxidized was used as a measure of cytoplasmic oxidation. It is expected that if the selected compounds could act as antioxidants, fluorescence will be inhibited compared to the positive control.

Hypothesis 3

Due to the presence of the catechol moiety and numerous hydroxyl groups, (+)-Catechin is expected to be acidic in nature. Based on these chemical attributes it is expected to react via hydrogen transfer reactions to neutralize ROS. (+)-Catechin should therefore have a high oxygen radical absorbance capacity (ORAC) value but low ferric reducing antioxidant power (FRAP) and Chelation values.

CHAPTER II
MATERIALS AND METHODS

Extract Preparation

D. bulbifera samples included tissues taken from bulbils acquired from a local, non-cultivated population. A common protocol in the literature is to dry the plant material in an oven. However, research shows that heat may reduce the overall phenolic concentration in *Dioscorea* species (Fang et al 2011). Therefore, in this study the plant material was sliced and then ground in liquid nitrogen to preserve the chemical state at time of collection. Prepared tissues were stored at -80°C for future use.

Plant extracts were prepared using the methods from Mikulic-Petkovsek et al 2015, with some modifications. Prepared plant tissues (2.5 grams) were combined with 25 ml of methanol and shaken at 800 cycles per minute for 1 hour in a dark refrigerated chamber. The resulting extract was centrifuged at 10,000 rpm for 10 minutes. Unwanted plant debris was discarded, and the supernatant was collected and acidified to 0.1% with formic acid to preserve acidic components. A liquid-liquid extraction using a 1:1 volumetric ratio of hexane to methanolic extract was performed to remove hydrophobic components that might interfere with compound identification. The methanol-hexane mixture was shaken at 800 cycles per minute for 20 minutes in a refrigerated chamber then centrifuged at 10,000 rpm for 15 minutes. The hexane layer was removed and discarded. Due to compound concentrations being below instrument detection limits, the remaining methanolic extract was concentrated to 10X the original concentration. This was accomplished by dehydrating the extract in a vacufuge and resuspending it in 100 μL of methanol per 1 mL of original extract. The resulting solution was filtered through a 0.2 μL syringe filter system and used for testing.

Identification and Purification of Compounds

Bulbil extracts and a mix of standard phenolic compounds were analyzed using ultra high-performance liquid chromatography, diode array detector, electrospray ionization, and quadrupole time of flight mass spectrometry (HPLC-DAD-ESI-QTOF-MS). Standards used included the following: kaempferol, trans-ferulic acid, chlorogenic acid, caffeic acid, sinapic acid, benzoic acid, 2-coumaric acid, naringenin, p-coumaric acid, gallic acid, quercetin hydrate, and catechin hydrate. The instrument used was a Waters Acquity SDS. UHPLC-UV was performed using a Waters Symmetry C18 analytical column (150 mm x 4.6 mm, 5 μm i.d.). The flow rate was 0.75 mL/min, with a 5 μL sample injection size. A linear mobile phase gradient consisting of A: acetonitrile and B: water, both acidified to 0.1% with formic acid was used; 0-12 minutes from 12-85% A, 12-13 minutes hold at 85% A, 13-13.5 minutes from 85%-15%, ending with a hold from 13.50-16.50 minutes at 20% A to clear any remaining compounds from the column*. UV absorbance was measured at 280 nm at 20 points per second.

MS procedures were done in negative ion mode. The operating parameters were set as follows: Capillary voltage 10 eV, source temperature 95°C, scan range from 150 to 1300Da, 3 scans per second, lock mass of 1266.988 Da which corresponds to a common formate adduct seen in the background of negative mode scans using formic acid. Nitrogen was used as the carrier gas with the flow set to 600 L/hr. MS/MS was performed on the same instrument in data dependent mode, the top 3 peaks were selected for MSMS fragmentation every 3 seconds. The collision energy was ramped up from 10 to 30 eV over the course of 1 second. All other parameters remained the same. The resulting data was processed using MZ Mine 2.0 available as a free download online. Processing included the use of the following: centroided mass detection, ADAP chromatogram

builder, peak extender, duplicate peak filter, join alignment, isotope peak grouper, searches for common fragments, adducts, and complexes, and gap filling.

To compare the extract and the standard mix, the two peak lists were aligned, de-gapped, and duplicate peak filtered using MZ Mine 2.0. Retention times and m/z ratios were compared for similar peaks. Possible matches were identified as those with a retention time within 0.05 seconds and a m/z within 0.05 of the standard compounds.

The extract peak list was organized by descending peak height and the top 10 peaks became the focus of compound identification. A literature search was done for all compounds previously identified in *D. bulbifera* and related species. The Human Metabolome Database (HMDB) (Wishart et al 2018), Mass Bank of North America (MONA) (MassBank of North America 2018), and the Phenol Explorer database (PE) (Rothwell et al 2013) were used to find potential compound identities for the remaining peaks. After the compounds were identified, additional literature reviews were performed to select those that were understudied within the context of the goals of this project.

The selected compounds of interest were isolated and purified from the whole extract using an Agilent 1260 high pressure liquid chromatography instrument with diode array detector (HPLC-DAD). Like the previous UPLC-UV analysis, a unique linear gradient of A: acetonitrile and B: water, both acidified to 0.1% with formic acid was established for each compound using the same Waters symmetry C18 column. LC-DAD parameters were set as follows: The flow rate was 0.75mL/min, with a 5uL sample injection size, and column heat was set to 35°C. UV absorbance was measured at 280nm at 20 points per second. These isolates were dehydrated and subjected to Nuclear Magnetic Resonance (NMR) analysis for identity confirmation.

^{1}H and ^{13}C, correlation spectroscopy (COSY), and heteronuclear single quantum correlation spectroscopy (HSQC) nuclear magnetic resonance (NMR) studies were performed using a Bruker 400MHz instrument. The temperature was 30°C and the samples were dissolved in deuterated methanol. Rather than using tetramethyl silane (TMS), the residual methanol signal was used as an internal standard.

To determine the relative percent composition of each component in the samples, both the highest and lowest integration values for the known compound or main component (approximately equal to 1.0) were used. Each of the integration values of the unknown compounds taken from 6 to 7 ppm on the spectra were used to calculate individual percent compositions at the high and low main component level using the following equation: $Relative\ \%\ Composition = \frac{|Unknown - Main|}{Main} \times 100$. These values were then combined to define a range for each potential compound. Peaks with integration values within 0.10 were considered the same compound and were used to calculate a single percent composition range.

Estimating Concentrations and Percent Recovery

Preliminary analysis of the crude *D. bulbifera* extracts showed negligible amounts of quercetin. Additionally, Quercetin has a similar structure to many other phenolic compounds, making it a good candidate as an internal standard for assessing percent recovery through the isolation processing steps and estimating compound concentrations. According to PE, a database for phenolic compounds in plants, natural concentrations of quercetin range from 0.005 mg to 24 mg of quercetin per 100 g of plant tissue (fresh weight) (Rothwell et al 2013). This range was used to select concentrations to be used in the following procedures.

To estimate percent recovery of the extraction process, known quantities of quercetin hydrate were added to the bulbil tissues before extraction. Approximately 1 mg and 3.6 mg of

Quercetin hydrate per 2.5 g of bulbil tissues were added to separate vials, representing the high and low end of the natural range for this compound (see above). After extraction the spiked samples were concentrated, processed, and analyzed using the same HPLC-DAD instrument the same as the standard extracts. The only parameter that was changed was the addition of DAD scans at 260 nm due to concerns over poor absorbance of quercetin at the standard 280 nm wavelength. The initial and final quercetin concentrations of these vials, based on observed UV absorbance, were used in following equation to determine how much quercetin remained after extraction: $\left(\frac{Initial-Final}{Initial}\right) \times 100 = \%\, Recovery$.

Compound concentrations were estimated from a standard curve prepared from a 2:3 serial dilution of bulbil extract spiked with quercetin hydrate (range from 0.11 to 9.11 mg quercetin/g tissue). Each spiked sample was analyzed twice using the protocols listed in the liquid chromatography section. The average absorbance for each standard was plotted against the known quercetin concentrations and linear regression was performed. Average absorbances from four independent analyses of the extract compounds were compared to this curve after identification. This initial estimate was divided by ten to better represent the state of the original extract before concentration.

In Vitro Chemical Assays

A Ferric Reducing Antioxidant Power (FRAP) assay was used to test the reducing capacity of each sample by electron transfer (ET) reaction. In the presence of an ET antioxidant, Fe^{3+} is reduced to Fe^{2+} which reacts with ferrozine forming a magenta colored complex with a maximum absorbance at 490 nm. In this case, a large increase in absorbance equates to higher the antioxidant potential. The procedure followed a modified version of the methods from Santos et al 2017. The FRAP reagent was prepared just before use by combining the following reagents in a 10:1:1

volumetric ratio at pH 3.6: 300 mM sodium acetate buffer; 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) suspended in 40mM HCl; and 20 mM $FeCl_3 \bullet 6H_2O$. 180 μL of the FRAP reagent and 13 μL of the sample compound, or MilliQ water as a blank, were added to each well. Some compounds do not react to completion within the time limits typically applied in FRAP assays leading to misinterpretation of the antioxidant activity. Therefore, absorbance readings were measured every minute for 30 minutes (Pulido et al 2000; Huang et al 2005). This provided a more comprehensive look at the reaction kinetics and made it possible to confirm that the reaction was stable after 30 minutes. Eight concentrations of ascorbic acid, ranging from approximately 1 mg/L to 8 mg/L, and one blank were measured in duplicate a total of four times. The 30 minute absorbance values for all runs were averaged and normalized by the blank absorbance. The resulting normalized average absorbance value was used to plot a standard curve and linear regression was performed to compare the ascorbic acid standard values to that of a 4.2 μM (+)-catechin sample.

An iron chelation assay was performed to serve the following two functions: 1.) To gain information on the sample compounds preventative antioxidant capacity; 2.) As an indication of the level of interference in the FRAP assay due to chelation of the iron ions. The iron chelation assay followed the procedures from Liu et al. 2015 modified for 96-well plates. The basic protocol consisted of reacting an acidic solution (pH 4.9) of 145 μL of 0.1 M sodium acetate buffer, 4 μL of 2 mM $FeCl_2$, and 36 μL of the sample compound (or MilliQ water as a blank). After 5 minutes, 15 μL of 5 mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) was added to the solution and the reaction was monitored spectrophotometrically at 490 nm. A series of eight different concentrations of disodium ethylenediaminetetraacetic acid (EDTA) were used to create a calibration curve. EDTA does not

readily dissolve at neutral pH, so NaOH pellets were added to raise the pH to 8 when creating the initial 0.5 M stock solution. Water adjusted to pH 8 with NaOH pellets was used for all dilutions to ensure the EDTA remained in solution.

An Oxygen Radical Absorbance Assay (ORAC) was performed to assess the capacity of the sample to neutralize peroxyl radicals and break the chain of oxidative reactions. The mechanism tested in the ORAC assay is radical inhibition through hydrogen atom transfer. The procedure followed the methods listed in Lopez-Alarcon and Lissi, 2006 using the pyrogallol red (PGR) probe, with modifications for use with a microplate reader per Ortiz et al, 2011. The basic idea is that an antioxidant which can neutralize the peroxyl radicals will prevent the probe from being oxidized, preventing a sharp decrease in absorbance. Stock solutions of 1.0 x 10^{-4}M pyrogallol red (PGR) and 120 mM 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were prepared daily in 75 mM phosphate buffer, pH 7.4. The AAPH and a reaction mixture consisting of 10 μL PGR (5 μM final concentration) and the sample (50 to 500 μM) were incubated separately for 30 minutes at 37 °C. Then 17 μL AAPH (10mM final concentration) was added to the reaction mixture and the absorbance was measured every minute for 180 minutes at 490 nm. ORAC values were determined by integrating the area under the observed curve.

Cell Culture and Cell Based Assays

Human PLB-985 cells (also known as HL-60) are a promyeloblastic leukemia cell line. Prior to use they were terminally differentiated into mature neutrophils by adding 1.3% DMSO to complete media seeded with 0.13 x 10^6 cells/mL for 6 days (Cavnar et al 2011). The differentiated cells were then cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, penicillin and streptomycin (both 1X). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 (Cavnar et al., 2011).

Cellular antioxidant activity assays were performed using the CellROX Orange flow cytometry assay kit from Thermo Fisher Scientific. In addition to the CellROX Orange dye, the kit includes the following: tert-butyl hydroperoxide (TBHP) as a positive control; N-acetylcysteine (NAC) as a negative control; and SYTOX Red Dead Cell Stain. The CellROX Orange dye is colorless until oxidized, then it exhibits an excitation and emission maxima at 545 nm and 565 nm respectively. Decreased fluorescence indicates that the sample compound can prevent oxidation of the dye within the cytoplasm. Increased fluorescence of the red dye at 658nm indicates an increased number of dead cells presumably due to increased oxidative stress. Used as described this kit makes it possible to distinguish between healthy cells with negligible fluorescence, stressed fluorescent cells, and dead cells which are dyed red.

Samples and controls are outlined in Table 1. The cells and reagents were prepared as summarized in Table 2, all incubations were done at 37°C maintained at 5% CO_2 in the dark.

Table 1. List of controls and samples used in the cellular antioxidant activity assays.

Compounds	**Sample Type**
Water only	Blank
TBHP only	Positive Control
TBHP and NAC	Antioxidant Control
TBHP and 50 mM (+)-Catechin	Extract Sample
TBHP and 20 mM (+)-Catechin	Extract Sample
TBHP and 5 mM (+)-Catechin	Extract Sample

Table 2. Reagent preparation for CellROX dye kit.

Reagent/ Substrate	**Initial Concentration**	**Final Concentration**	**Amount (μL)**
PLB-60 Cells	3.5 x 10^6 cells in 700 μL complete RPMI growth medium	0.50 x 10^6 cells per reaction tube	100.0
TBHP	70% (7.78 M)	50 mM (2 μL stock to 330 μL RPMI)	4.0
NAC	10 mg dehydrated	250 mM (Reconstitute with 245 μL PBS)	4.0
CellROX Green	25 mM	500mM (3.6uL of CellROX with 29.4uL DMSO)	4.0
SYTOX Red	5 μM	N/A	N/A
(+)-Catechin	1.46 mM	50 mM	34.0
(+)-Catechin	1.46 mM	20 mM	14.0
(+)-Catechin	1.46 mM	5 mM	1.4

RPMI media (900μL), the selected sample compound or control (Table 2), and the cell solution were added to labeled microcentrifuge tubes and incubated for four hours. After incubation TBHP was added and the tubes were incubated for another hour. CellROX Orange dye was added and incubated for 1 hour.

Owing to an instrument failure, flow cytometry could not be completed. Instead, after staining (2x concentration of dye) the fluorescence of 200 μL of each sample was measured using a 24-well plate reader (530/590 nm excitation and emission). SYTOX Red was omitted to prevent overlapping absorbance with the CellROX Orange dye. Due to the expected variability of this type of instrument, and the low level of fluorescence, each sample was measured four times per run and the average was normalized using observed average absorbance of the blank.

CHAPTER III
RESULTS

Liquid Chromatography and Mass Spectrometry

MS measurements were originally performed on September 22, 2017. However, these samples eventually oxidized and were re-made. Due to seasonal metabolic variation, a change in chemical constituents was expected in the new extract and was confirmed during preliminary LC analysis (Figure 1). Additionally, it was discovered that an instrument issue resulting in increased pressure may have distorted the original retention times. Consequently, the MS measurements were repeated on January 31, 2018 (Figure 2). Upon comparison, several differences were noted. The shift in retention times was confirmed with compounds reaching the detector as much as 0.7 minutes later in the newer spectrograph. Although many of the same peaks were observed, their relative peak heights changed dramatically. The January data was selected for use in the rest of the study except for comparing reference standards to possible trace compounds.

Before the issue with extract oxidation, the September data was compared to nine standard reference compounds (Table 3). Six of these compounds were identified as possible constituents in the *Dioscorea* extract including the following: (+)-catechin, quercetin, sinapic acid, 2-coumaric acid, *p*-coumaric acid, and trans-ferulic acid. (+)-catechin had a peak height of 60,035 making it the third most abundant compound in the extract. The other compounds had relative peak heights ranging from 3 to 46. Cumulatively these compounds (excluding (+)-catechin) comprised approximately 0.06% of the total chemical population in the September 2017 data and were not re-evaluated in the January 2018 run. The results of this analysis are summarized in Table 4 below.

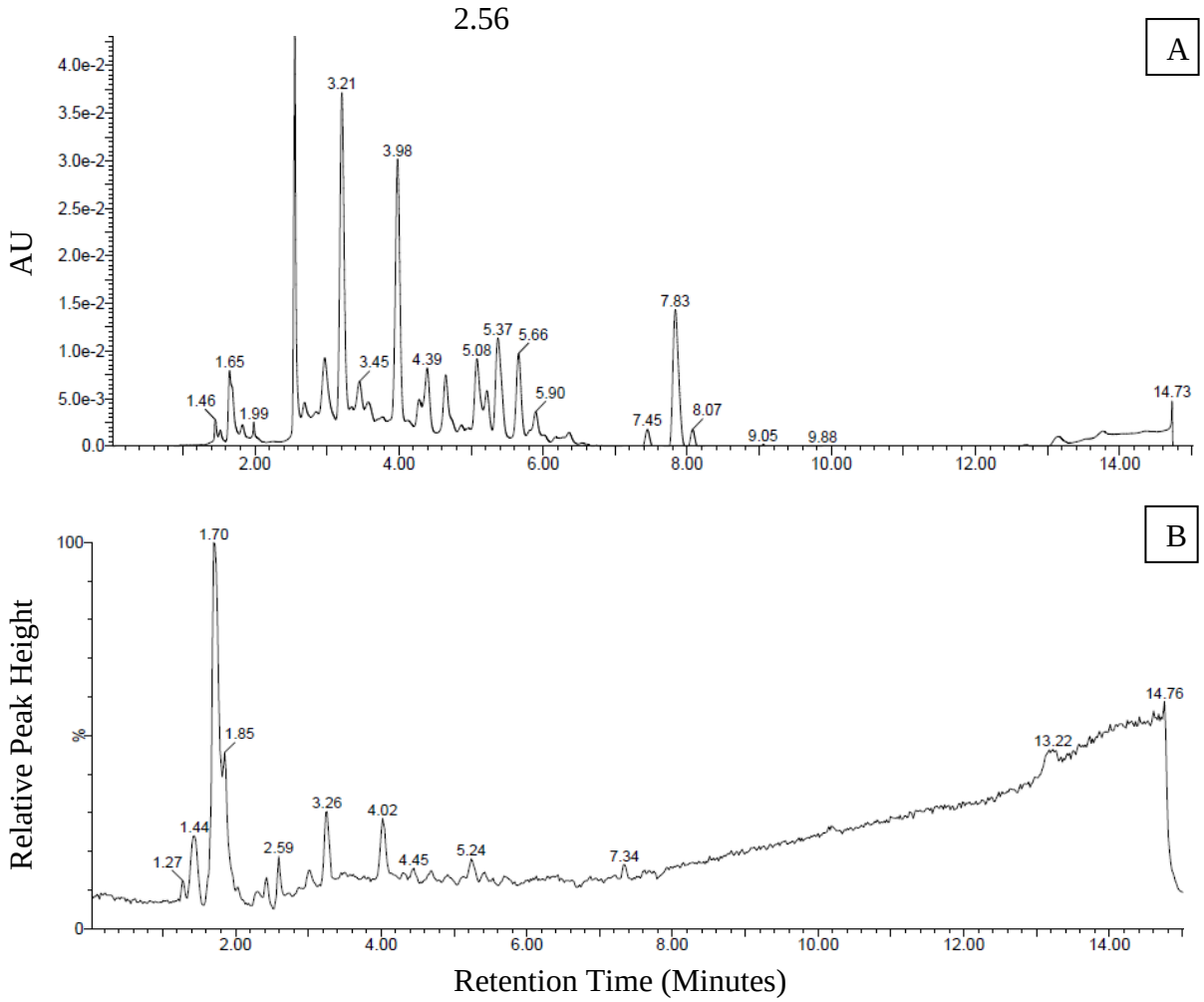


Figure 1. LC (A) and MS (B) data from September 22, 2017.

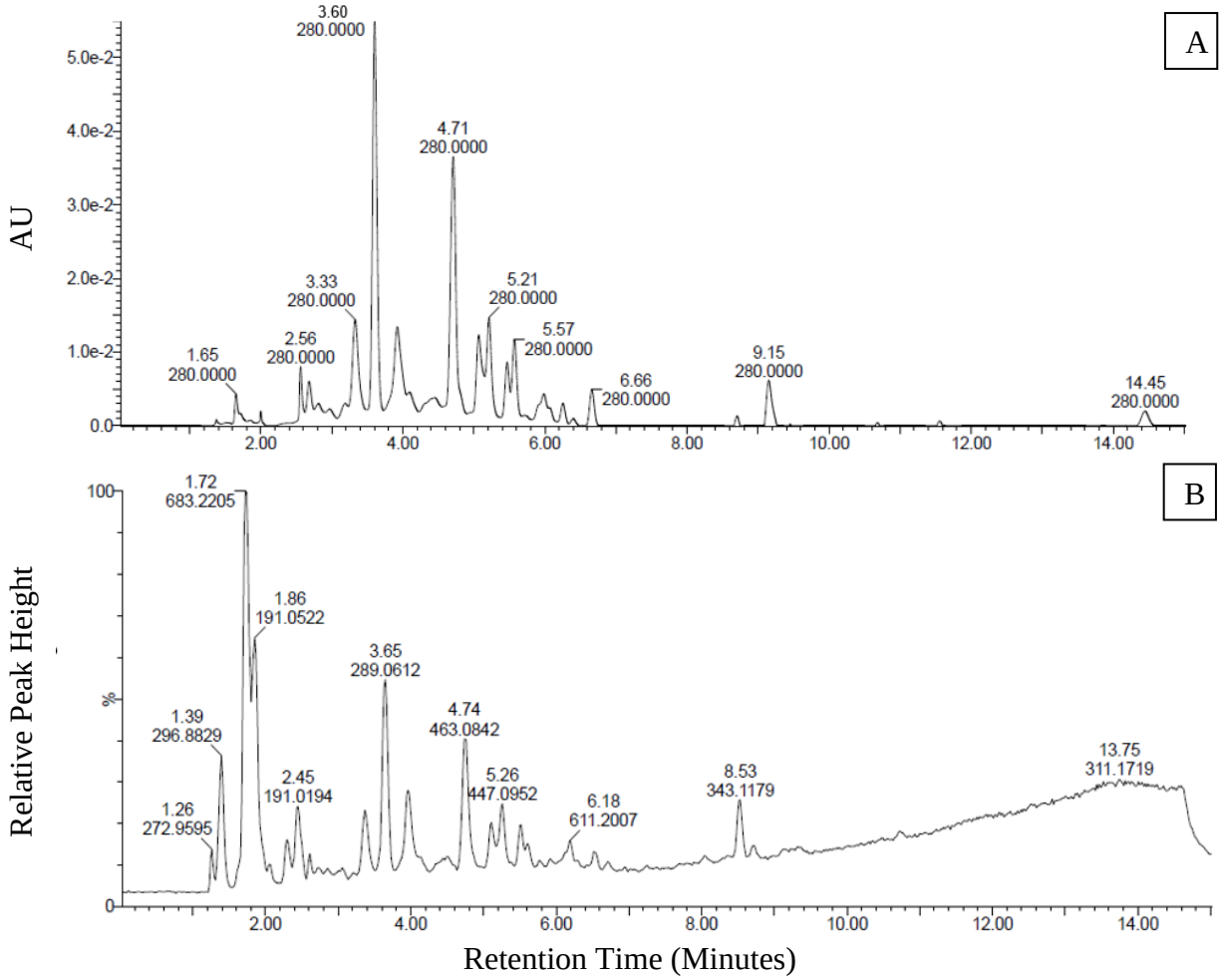


Figure 2. LC (A) and MS (B) data from January 31, 2018.

Table 3. Comparison of extract peaks to standard compounds during UHPLC-UV-MS analysis, by order of descending peak height.

Compound Identity	Average m/z	Average Retention Time (min)	m/z Difference (Standard vs. Extract)	Retention Time Difference	Possible Match (Y/N)
Catechin	289.060	3.639	+0.005	-0.034	Y
Quercetin	301.024	6.647	+0.019	+0.001	Y
(+)-Naringenin	271.063	7.599	-0.007	+0.115	N
Chlorogenic Acid	353.083	3.132	+0.005	-0.183	N
Caffeic Acid	179.033	3.921	+0.000	+0.097	N
Sinapic Acid	223.064	4.787	-0.007	+0.046	Y
2-Coumaric Acid	163.038	5.853	+0.001	+0.034	Y
Trans-ferulic Acid	193.050	5.001	-0.004	-0.017	Y
p-Coumaric Acid	163.037	4.810	+0.003	+0.000	Y

Using MZMine 2.0, a new peak list was generated using the January MS data. Based upon their relative heights the top ten peaks were selected for identification and are listed in descending order of abundance in Table 4. Peak 1 was tentatively identified as a catechin isomer based on comparison to a reference standard. Peak 1 also had characteristic fragments at m/z 245, 205, 203, 125 as seen in the literature (Souza de Silva et al 2017) and in spectra from MONA. Based on the MSMS fragmentation, peak 9 is likely a dimer of peak 1. It eluted just 0.009 minutes after Peak 1, the base peak was m/z 289.06, and it had the same fragmentation pattern as peak 1. Peak 2 was identified by the MZMine software as a possible dimer of m/z 341.11. No potential matches were found during the database or literature search, so this could not be confirmed. Peak 3 was tentatively identified as a quercetin derivative based on the characteristic peaks at 301, 300, 271, 255, 179, 151 seen in the literature (Yang et al., 2013, Li et al., 2016, Zhu et al., 2017) and comparison to spectra in MONA. The database search for Peak 4 returned several sugar compounds, but the exact compound could not be determined based on the

observed ion fragments. Peaks 5 through 8 either returned no results in the database search, were not selected for MSMS fragmentation, or were the product of other ions. Peak 10 was tentatively identified as a type B procyanidin dimer in the database search. The literature supported this conclusion based on the fragments at m/z 577, 451, 425, 407, and 289 (Kolniak-Ostek, Oszmiański 2015; García-Salas et al 2015; Souza de Silva et al 2017; Chen et al 2018). The exact compound could not be elucidated because the spectra for these compounds are so similar. Based on these findings Peaks 1 and 3 from the MS data were selected for NMR studies.

Table 4. Summary of the January MS analysis.

Peak #	Tentative Identification	RT	Exp. m/z	MS Level	MS^2 Fragments (by order of decreasing abundance)
1	Catechin isomer	3.639	289.060	2	289.058, 245.071, 109.023, 123.038, 125.018, 203.062, 151.032, 205.042
2	m/z 341.11 Dimer	1.724	683.222	1	341.105, 387.115, 1025.352, 729.237, 474.150
3	Quercetin derivative	4.749	463.086	2	463.069, 300.015, 301.022, 464.072, 302.028, 465.068, 271.013, 255.015, 150.999, 178.986
4	Unknown Sugar	1.729	341.106	2	149.042, 341.101, 101.021, 191.052, 179.052, 71.012, 113.022
5	Undetermined	1.872	191.052	2	191.047, 85.023, 111.002, 87.003, 93.028, 192.051, 127.032
6	Undetermined	1.729	387.111	2	Not Selected
7	Undetermined	4.009	561.142	2	289.060, 561.116, 407.060, 290.063, 435.089,562.122, 125.017
8	Possible Artefact	1.821	157.034	2	Alternate ion selected which yielded this ion
9	Catechin Dimer	3.648	579.153	2	289.068, 112.983, 290.076, 579.147, 115.001, 580.154, 577.134
10	Procyanidin Type B Dimer	3.627	577.137	2	407.061, 289.060, 577.112, 425.071, 578.116, 125.0171, 426.074, 451.076

Nuclear Magnetic Resonance

MS Peak 1 was identified as a mixture containing (+)-catechin, possible traces of epicatechin based on comparison to spectra in the HMDB and the observed NMR data (Figures 3 through 5 and Tables 5 and 6). HSQC could not be interpreted because the sample was too dilute to give sufficient signal to outcompete the background noise.

The highest and lowest integration values for (+)-catechin were 1.00 and 0.95. Integration values for the unknown(s) (0.66, 0.40, 0.35, and 0.22) indicate as many as 3 additional compounds within the sample. Relative to (+)-catechin, unknown 1 (integral 0.66) had a range from 66 to 69%, unknown 2 (integrals 0.40 and 0.35) had a range from 35 to 42%, and unknown 3 (integral 0.22) had a composition range of 22 to 23%. The other components of the sample could not be identified.

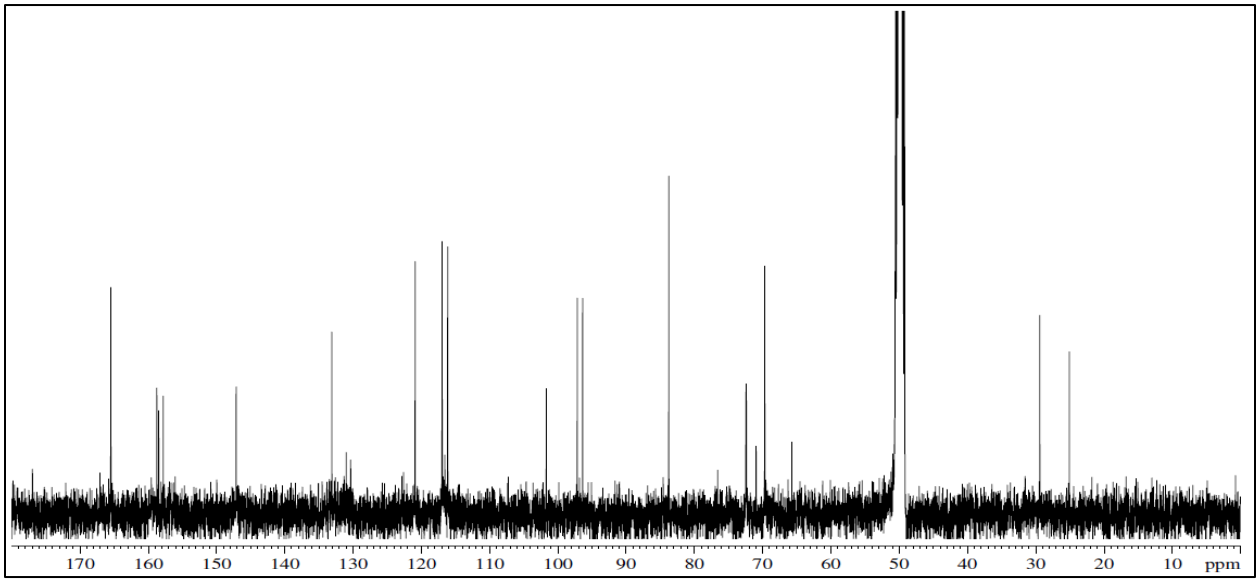


Figure 3. Carbon NMR for (+)-catechin.

Table 5. Chemical shifts for the C-NMR of (+)-catechin.

Associated with (+)-Catechin	29.40, 65.72, 83.73, 96.3, 97.14, 101.68, 116.12, 116.93,120.89, 130.98, 147.10, 157.79, 158.45, 158.72, 165.47
Associated with Unknown(s)	25.07, 70.95, 72.40, 120.89, 133.09
Solvent	49.22-50.50 (9 peaks)

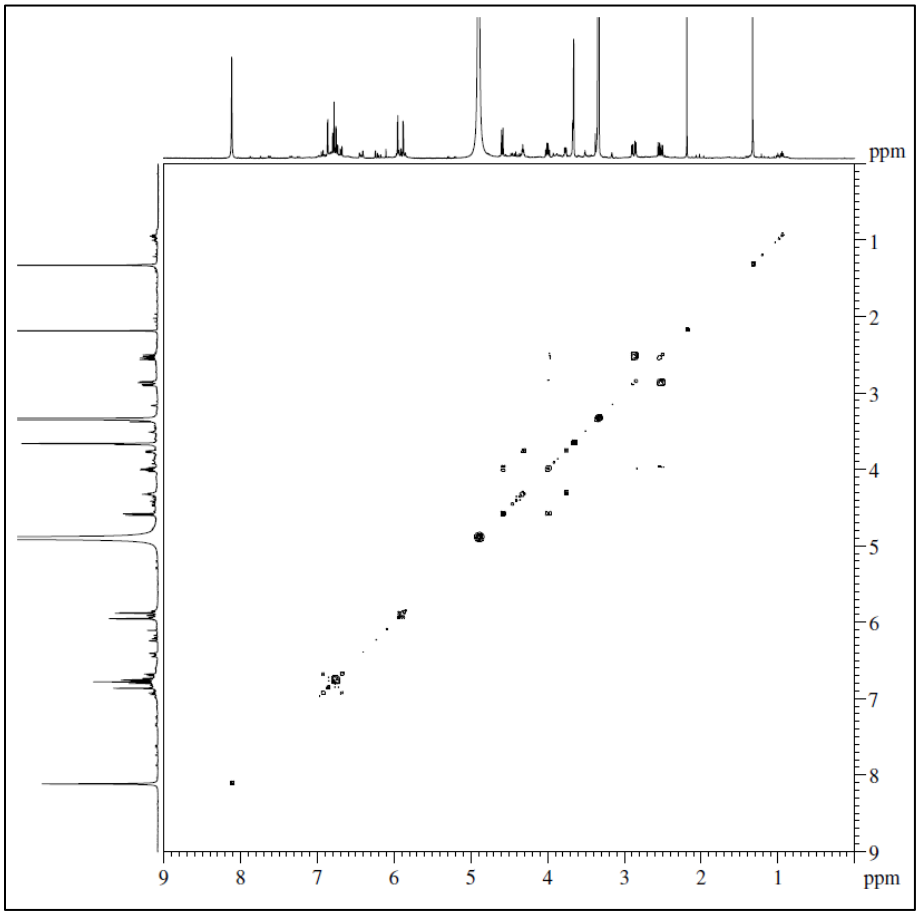
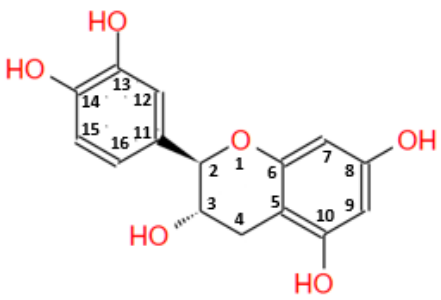


Figure 4. COSY NMR for (+)-catechin.

Table 6. H-NMR chemical shifts for (+)-catechin.

Atom	# Protons	Chemical Shift (ppm)	Coupled Atoms
4	2	2.53 (q), 2.88 (q)	3
3	1	4.00 (q)	2, 4
2	1	4.59 (d)	3
9	1	5.88 (d)	7
7	1	5.94 (d)	9
16	1	6.74 (t)	15, 12
15	1	6.77 (d)	16, 12
12	1	6.86 (d)	16, 15



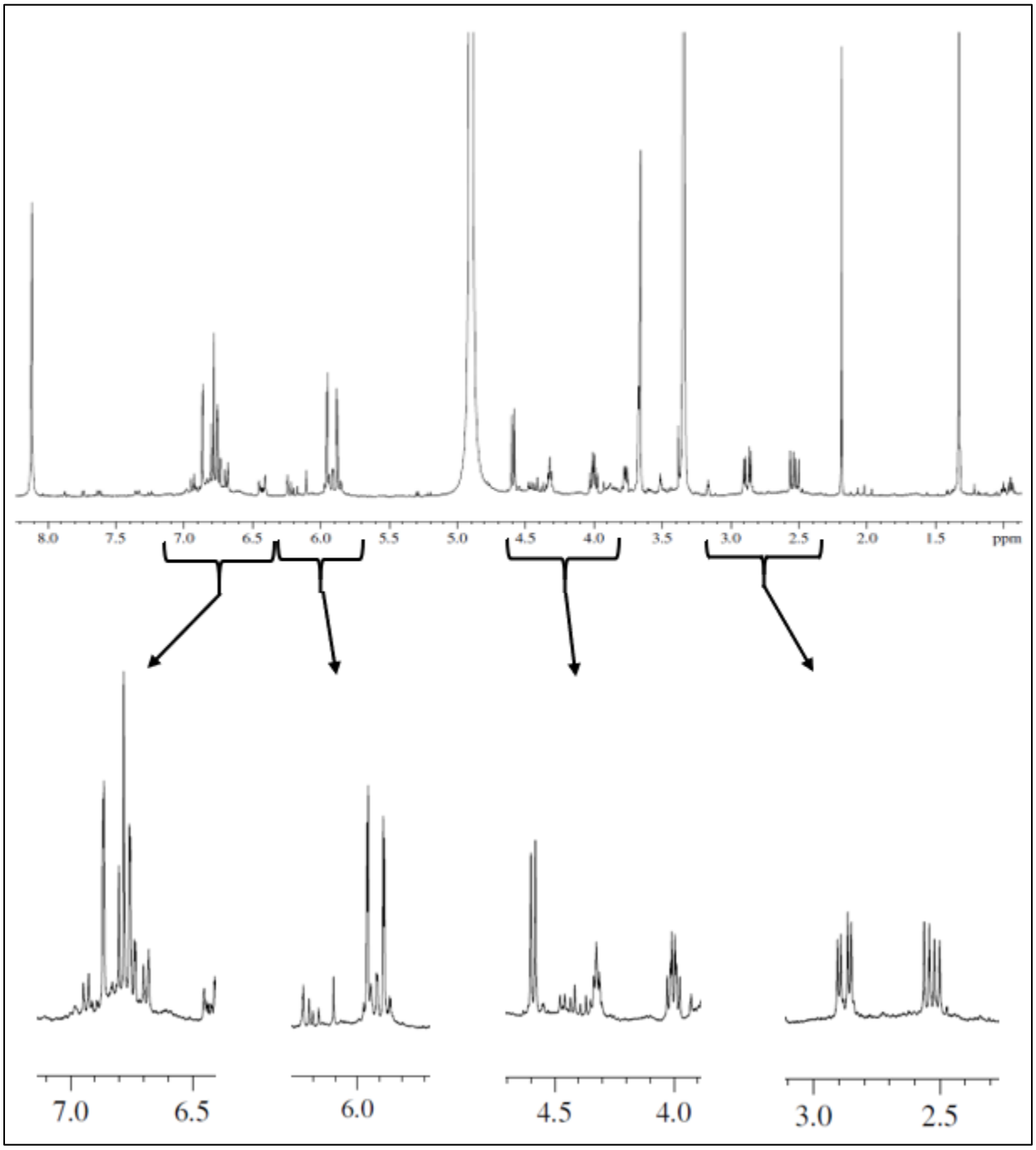


Figure 5. Proton NMR for (+)-catechin.

MS Peak 3 could not be positively identified due to the complexity of the spectra and the number of co-eluting compounds in the sample (Figures 6 through 8 and Tables 7 and 8). Again, the HSQC could not be interpreted because the sample was too dilute to give a strong signal above the background noise. The H-NMR spectra for MS Peak 3 gave thirty-four different integration values ranging from 0.11 to 5.43. By grouping the values that were within 0.10 of each other, five groups were identified. This suggests the presence of five compounds in the sample. Many other integrals remained ungrouped, so additional compounds are possible.

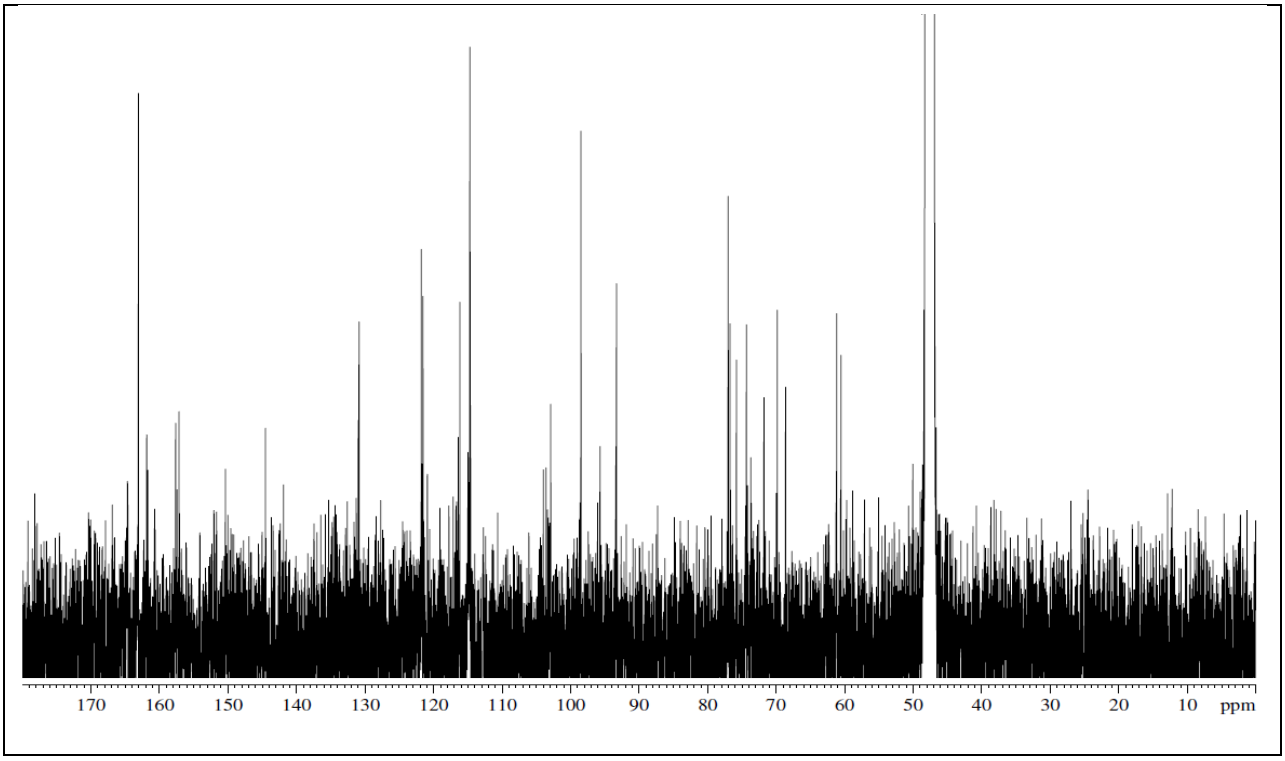


Figure 6. Carbon NMR for MS Peak 3.

Table 7. Chemical shifts for the C-NMR of MS Peak 3.

Associated with Peak 3	60.55, 61.17, 68.63, 69.84, 71.78, 74.33, 75.80, 76.73, 77.00, 93.10, 98.49, 102.90, 114.61, 114.68, 116.15, 121.54, 121.79, 130.88, 157.14, 163.07
Solvent	46.95-48.30 (10 peaks)

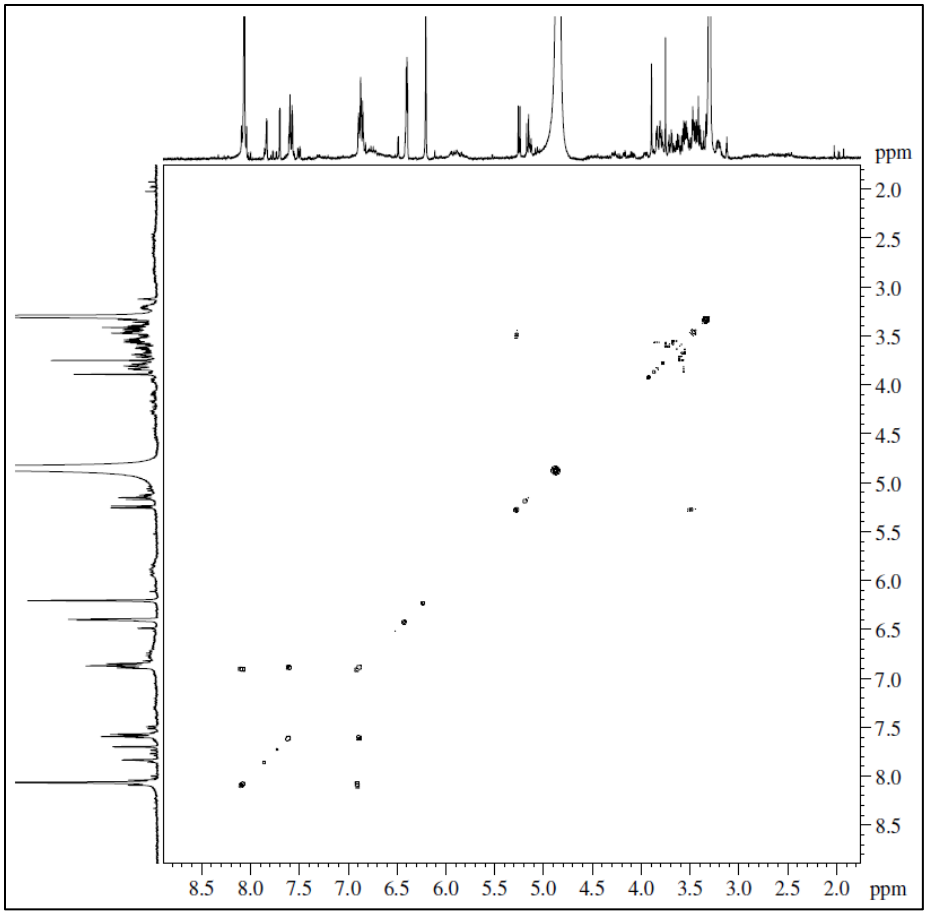


Figure 7. COSY NMR for MS Peak 3.

Table 8. H-NMR Coupling Data for MS Peak 3. Bold indicates areas of coupling.

Chemical Shift (ppm)	Approximate Chemical Shift of Coupled Groups
0.941, 0.924, 1.306, 1.955, 1.997, 2.009, 2.052, 3.239-3.257, 3.324-3.336, **3.420-3.504**, 3.554, 3.599, 3.719, 3.779, 3.814, **3.834-3.839**, **3.865**, **3.873**, **3.922**, 5.152-5.197, **5.263-5.282**, 6.230-6.235, 6.420-6.433, 6.514-6.519, **6.876-6.926**, **7.596-7.601**, 7.617-7.634, 7.725-7.630, 7.856-7.861, **8.063**	**3.4, 3.8, and 5.3** **6.9, 7.6, and 8.1**

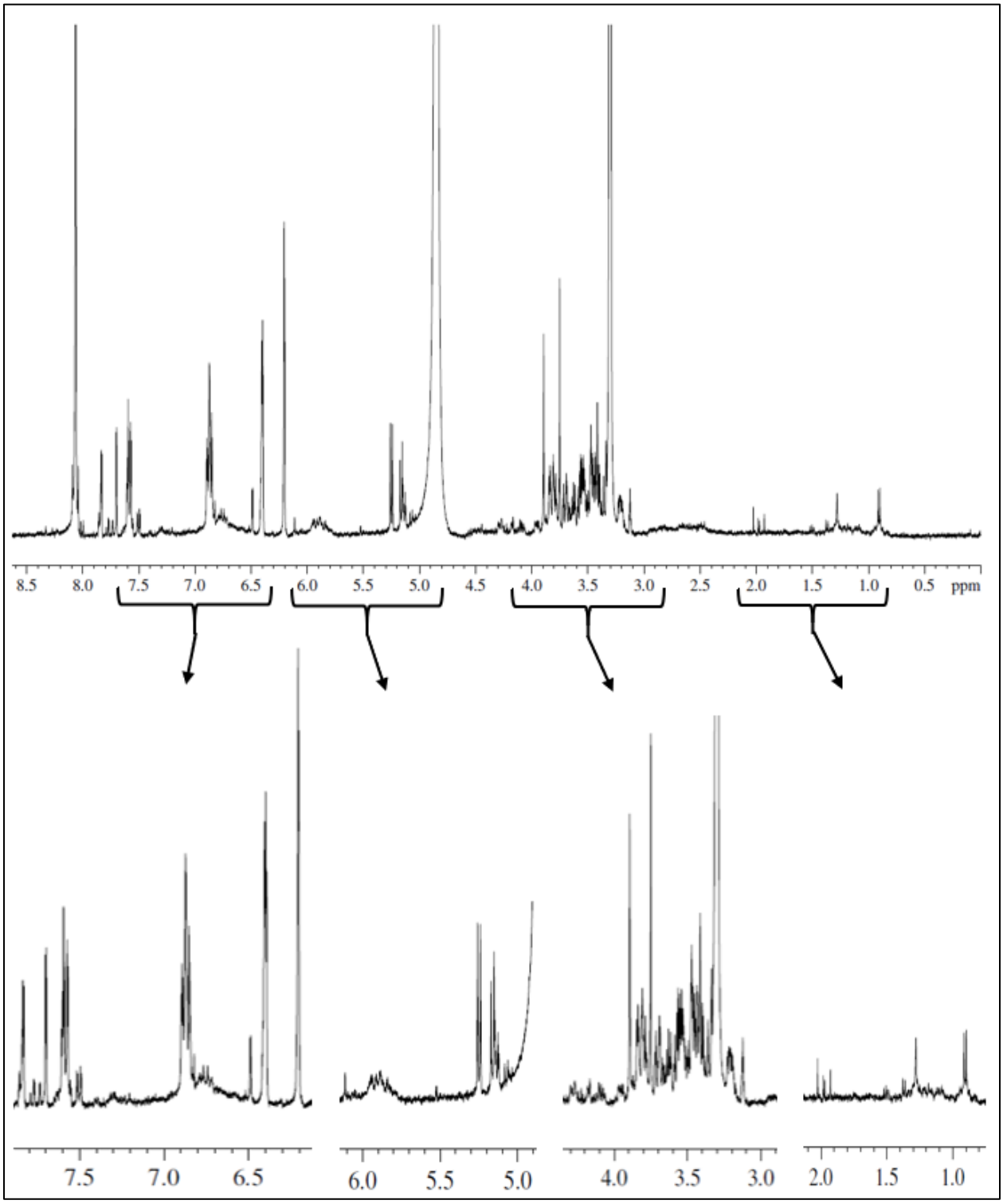


Figure 8. Proton NMR of MS Peak 3.

Estimated Compound Concentrations and Percent Recovery

Linear regression performed on the quercetin dilution series yielded the equation y=0.0183x-0.2488 with an R^2 value of 0.991. The equation was used to estimate the quantity of (+)-catechin and the MS Peak 3 compound in the extract. It was also used to estimate the percent recovery of the extraction process. This data is summarized in Figure 9 and Tables 9 and 10 respectively. Table 9 also includes adjusted quantities based on the estimated percent lost during extraction. After approximately 625 mAU (equivalent to 20.50 mg/g of tissue), the standard solution became saturated and the absorbance readings became erratic. Unfortunately, the high dose percent recovery vial had an absorbance reading exceeding this (average 896 mAU) so it could not be reliably quantified.

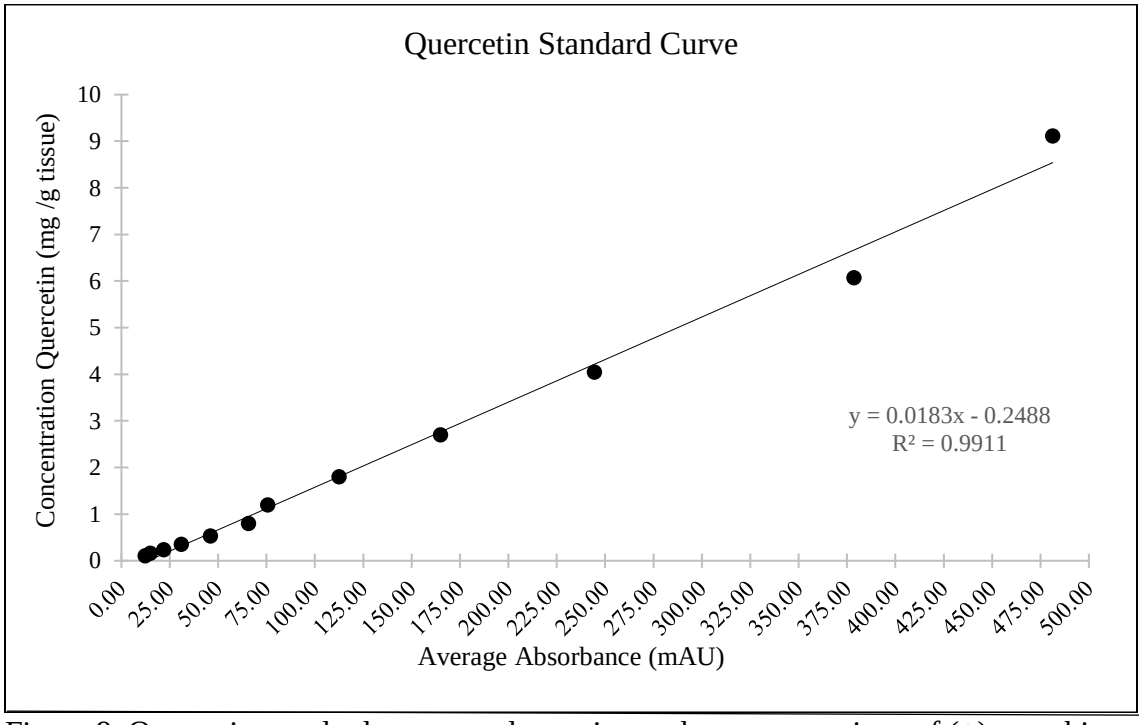


Figure 9. Quercetin standard curve used to estimate the concentrations of (+)-catechin and Peak 3 in the *D. bulbifera* extract.

Table 9. Estimated Concentrations Based on Internal Standard.

Compound	Estimated Concentration Range (mg/g tissue)	Adjusted Range (mg/g tissue)
Catechin	1.25-2.58	2.09-4.30
MS Peak 3	4.88-4.91	8.15-8.19

Table 10. Estimated Percent Recovery.

Quercetin Added (mg/g)	Amount Recovered	Percent Recovery
0.40	0.53	33.01
1.44	N/A	N/A

FRAP

Figure 10 shows the results of the standard curve produced using eight concentrations of ascorbic acid (Ranked in decreasing concentration and denoted as Treatments 1 through 8) and one blank (Treatment 9). Linear regression generated the equation $y = 0.0488x+1.0127$ with an R^2 value of 0.9968. Based on this linear regression, 4.2 μM (+)-catechin with a normalized average absorbance of 1.183 is approximately equivalent to 1.070 mg/L of ascorbic acid.

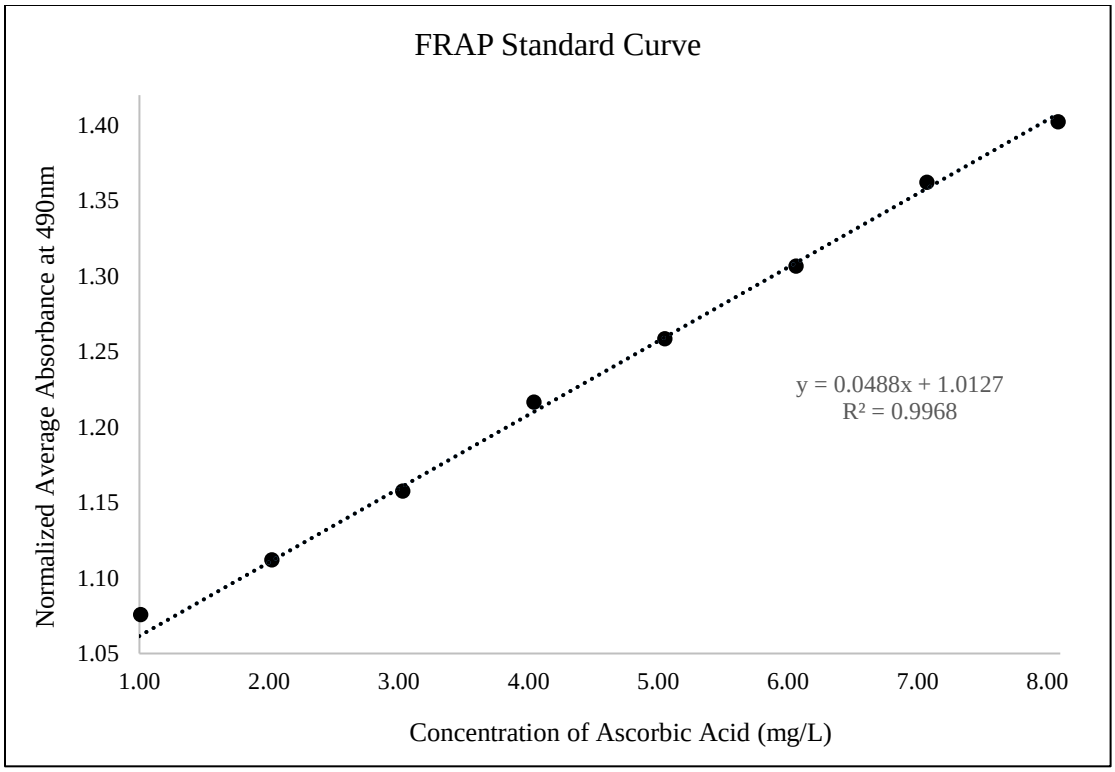


Figure 10. FRAP standard curve using ascorbic acid.

Iron Chelation

Figure 11 shows the results of the standard Na_2EDTA curve with the equation $y = -0.0737x + 0.4425$ and an R^2 value of 0.9216. Although this test was run many times an R^2 value greater than this was not achieved. However, this was deemed of little significance because despite the use of increasingly concentrated (+)-catechin samples, the absorbance for this compound always fell outside the curve. In fact, its absorbance was consistently higher than the blank using only water.

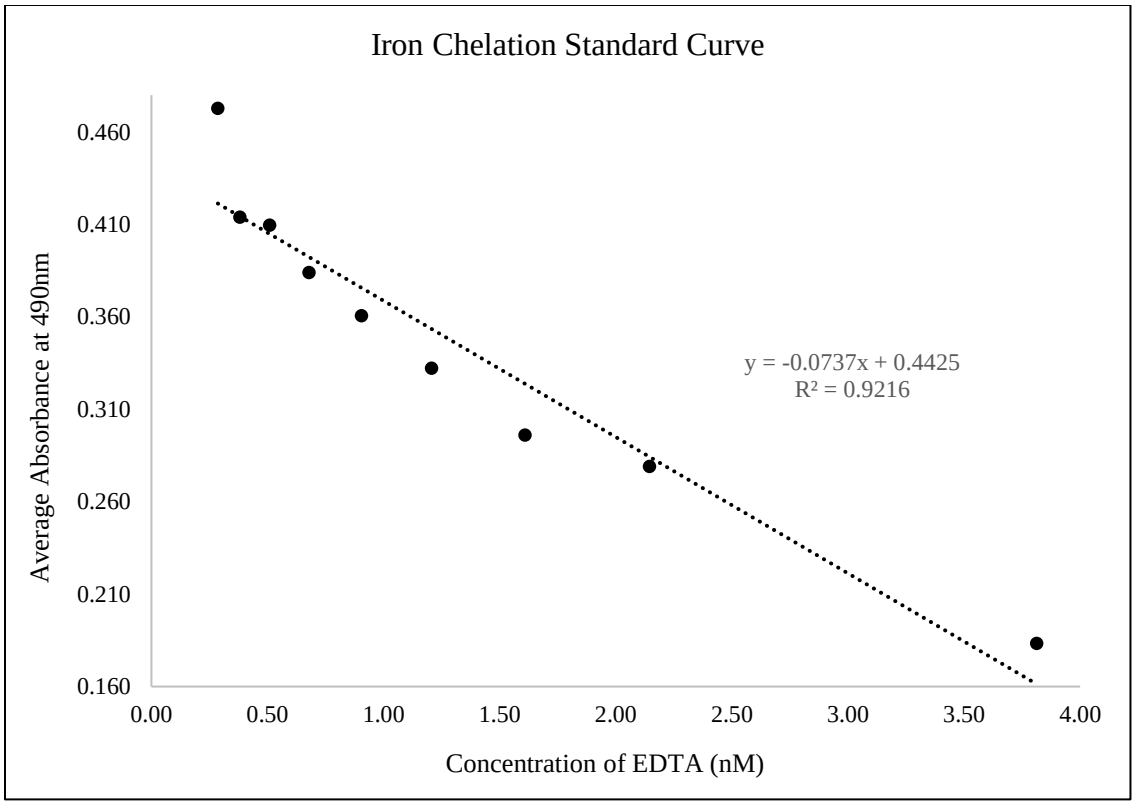


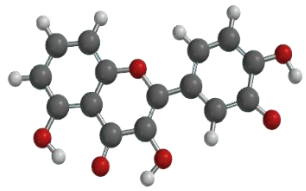
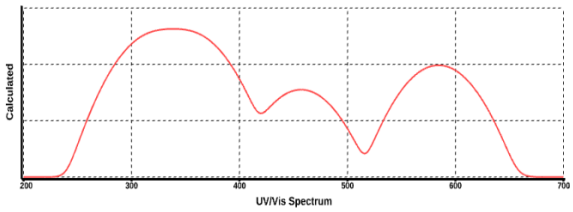
Figure 11. Percent inhibition of ferrozine complex formation by treatment number.

ORAC

The reaction between AAPH and (+)-catechin produced radical intermediates which absorbed light within the measured range. As a result, any possible decrease in absorbance of the PGR probe was overshadowed by the increasing absorbance of the catechin radicals. Spartan 2.0.6 software from Wavefunction Inc., Irvine, California, was used to assess the possible intermediates that could be formed as (+)-catechin reacts with AAPH. Four radicals were identified, and their UV-Vis spectra were predicted Figures 12A through 12J).

Each radical was assessed to determine which would be most prevalent and if there was an ideal wavelength to measure PGR decay without measuring radical absorption. Based on the calculated free energies, radical 2 would be the predominant product. Unfortunately, all the predicted radicals exhibited UV-Vis spectra which overlap with the desired measurement range.

Consequently, no ideal wavelength was identified that would allow for measurement of (+)-catechin using the ORAC assay.

 -1027.99781 au	
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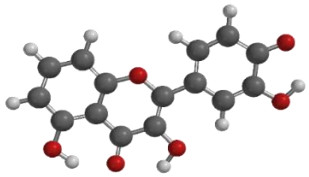
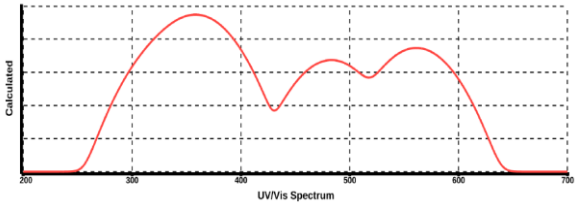
 -1028.00085 au	
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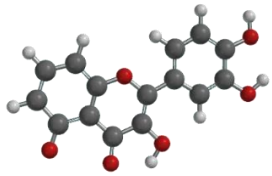
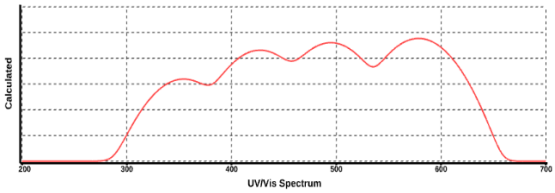
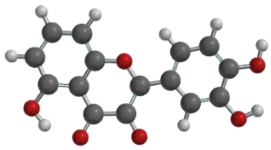
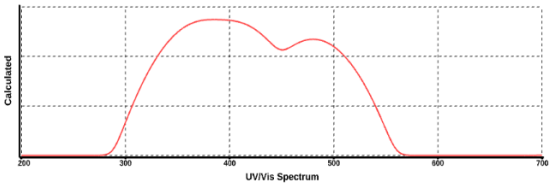
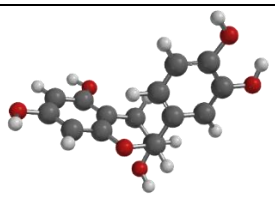
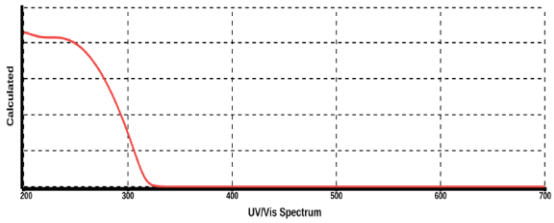
 -1027.96263 au	
E. Radical 3 Structure.	F. Radical 3 Spectra.
 -1027.98595 au	
G. Radical 4 Structure.	H. Radical 4 Spectra.
 -1031.01747 au	
I. (+)-Catechin Structure.	J. (+)-Catechin Spectra.

Figure 12. Structures and UV-vis spectra for the ORAC radicals and reagents.

Cellular Antioxidant Assays

Due to instrument malfunction only the positive/negative controls, NAC and 50 μM (+)-catechin samples were measured using flow cytometry (Figures 13A through 13C). In ascending order, fluorescence peaks of the CellROX orange dye was as follows: the blank, antioxidant control, 50 μM (+)-catechin, and the positive control. The average area of each peak is displayed in Figure 13C. SYTOX red results for the antioxidant control, the (+)-catechin and the positive control were very similar. All were greater than the blank (Figure 13B). No replicates could be completed, preventing any statistical analysis.

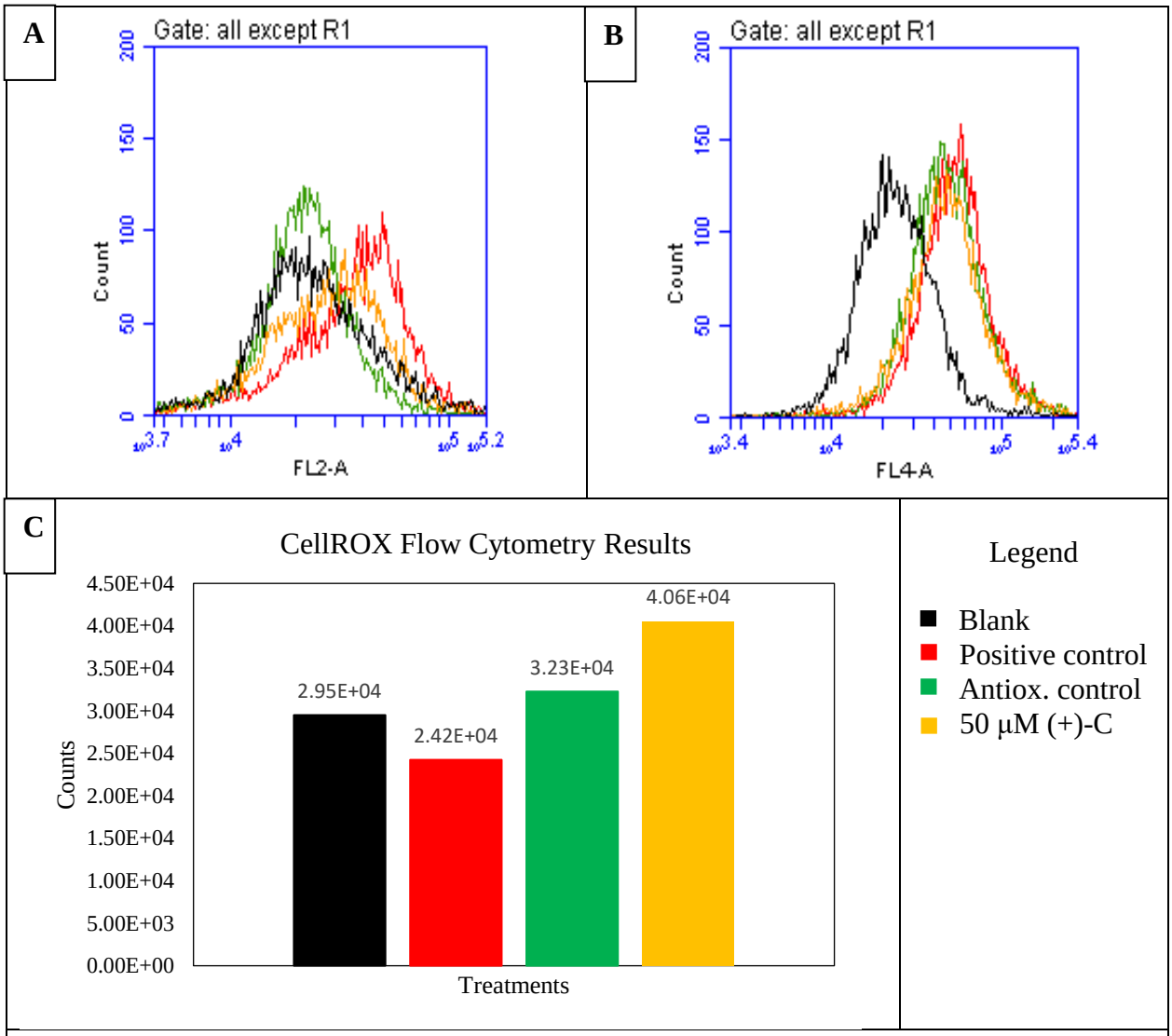


Figure 13. Flow cytometry data for all treatments. A: CellROX, B: SYTOX Red, C: Average peak area of CellROX data.

The data was further explored by measuring the differences in fluorescence (530 nm/590 nm excitation/emission) in 24-well plate reader using twice the dye concentration. Both the 20 µM and 5 µM (+)-Catechin samples (9.47E+4 and 9.47E+4) showed fluorescence levels higher than both the blank and the TBHP control (6.69E+4 and 7.81E+4 respectively). Again, the 50 µM (+)-catechin sample showed decreased absorbance (65716) compared to the positive control

but greater fluorescence than the negative control. Unlike the flow cytometry data, this time the 50 μM (+)-catechin had lower fluorescence counts than the blank. See Figure 14 for a summary of this data.

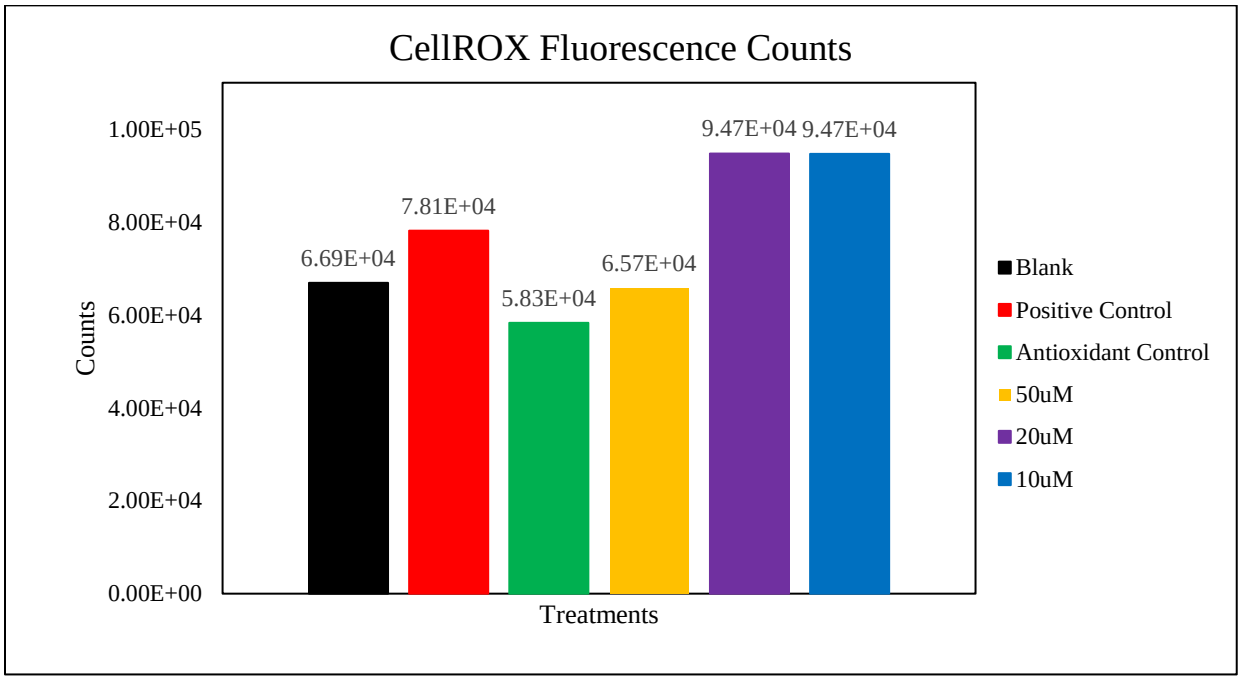


Figure 14. Fluorescence counts of the CellROX dye as measured using the 96-well plate reader.

CHAPTER IV
DISCUSSION

Liquid Chromatography and Mass Spectrometry

Initial setbacks with the LC-MS data, due to increased system pressures, were noted but deemed inconsequential. The retention times were skewed in the September 2017 data compared to the January 2018 runs. However, the timing between all the extract and the standard runs in September were consistent. The September data was therefore still relevant for identifying the trace components. Additional concerns were raised after the original extracts oxidized and had to be re-made. Plant metabolites vary greatly by season, so changes in the phenolic composition of the extracts were anticipated. Initial LC examination confirmed that both peak height and composition had changed in the new extract versus the original. However, the intent of this study was not to investigate the composition of the extract at a single point in time but rather to explore what compounds the plant can produce. As a result, proceeding with the identification of phenolic compounds using only the January data did not violate the intent of the study.

The discovery of (+)-catechin, quercetin, sinapic acid, 2-coumaric acid, *p*-coumaric acid, and trans-ferulic acid in the *Dioscorea* extract did not come as a surprise. Each of these standard compounds was selected either because it is very commonly found across numerous plant families or because it constitutes a significant step along the Shikimate Pathway used to synthesize precursor compounds for phenolic compounds (Dewick 2009). Because of their status as key intermediates in this pathway, it was also not surprising that most of them were present in trace amounts rather than as major components. Based on their concentration within the *Dioscorea* extract, quercetin, sinapic acid, 2-coumaric acid, *p*-coumaric acid, and trans-ferulic acid are not likely to contribute to any medicinal effects seen from this plant in their pure form. (+)-catechin on the other hand, represented the third highest peak in the September MS data and the top

component in the January data. The high concentration of catechins could explain the yellow color of the bulbil flesh and could contribute to any medicinal effects the plant may have. It is important to note that phenolic compounds commonly exist as derivates bonded with things like glucose and other sugars (Comert and Gokmen 2018). These compounds may not have been recognized during the comparison to the chemical standards. A more intensive MS study, including ESI+ mode, would need to be done to assess the full complement of compounds in the extract.

Other compounds in the January MS data include a possible quercetin derivative (Peak 3), an unknown sugar (Peak 4), possible dimers of the observed catechins, and a procyanidin type B dimer. These were tentatively identified based on their fragmentation patterns which closely resembled those found in the literature and in MONA or HMDB. Despite the close similarities between the published data and the MS data, there were some discrepancies in the observed ion fragments (missing peaks, varying fragment peak heights). There could also be issues due to the presence of isotopes with very similar m/z and fragmentation patterns not being recognized as separate compounds. To confirm the identities of these peaks, and to identify other compounds in the extract, more data would be needed. Additional chemical standards should also be analyzed, and samples should be run at a slower flow rate to achieve a better separation of isotopes and derivatives that may have led to inconsistencies in ion detection.

NMR

As a secondary identification method, the top 2 peaks tentatively identified in the January MS data, (+)-catechin and Peak 3 were selected for NMR experimentation. NMR samples for these peaks were collected by hand as they eluted from the LC. Because of this, the samples were very dilute and had to be concentrated by evaporating the solvent. In the end, the presence of (+)-catechin was confirmed and a lesser amount of epicatechin is suspected based on comparison with

published NMR spectra (MONA). The NMR data for MS Peak 3 was inconclusive. Quercetin derivatives are quite diverse and have very similar spectra in both MS and NMR studies. A higher quality, more concentrated sample would be needed to isolate specific spectral differences and make a positive identification for this sample. It is also important to note that due to the crude collection process, both NMR samples contained numerous co-eluting compounds. Based on integral calculations, both NMR samples contained at least three trace components that further complicated the analysis. Future studies should be done using an automated collection process to obtain cleaner samples. The heat used during the evaporation process could have degraded the phenolic components. Methods should be investigated for retrieval of a more concentrated samples so that evaporation is not required. Alternate drying methods such as lyophilization should also be explored.

Estimated Compound Concentrations and Percent Recovery

Linear regression of a quercetin dilution series yielded a strong linear relationship (Figure 9). Unfortunately, the percent recovery results were not as impressive, indicating losses of as much as 33% (Table 10). From this it was estimated that the *Dioscorea* extract contains between 1.25 to 4.30 mg of (+)-catechin per gram of bulbifera tissue, depending on if you use the raw estimate or the adjusted values based on percent recovery (Table 9). This very wide concentration range would need to be better characterized before *D. bulbifera* could be considered for medicinal uses. An even larger concentration range was seen with Peak 3 (4.88 to 8.19 mg/g) (Table 9), again indicating a high level of discrepancy in the data that should be addressed in further studies.

Percent recovery could possibly be improved by grinding the plant tissues finer, allowing the tissue to soak overnight in the MeOH, or using gentle heat. Keeping the extract cold could certainly have limited the extract efficiency. Still, the use of heat should be approached cautiously

as it could alter the chemical composition by degrading phenolic species. It is also possible that the chemical nature of quercetin and (+)-catechin and peak 3 differ enough that they do not respond the same to the extraction process. Standard curve trials should be done using compounds other than quercetin to assess this.

In vitro Antioxidant Studies

The FRAP assay is a common in vitro test used to assess antioxidant potential via electron/proton donation. A strong linear relationship was seen in the linear regression analysis of a standard curve using ascorbic acid, a well-known antioxidant compound. (+)-catechin showed antioxidant potential based on the FRAP assay, being approximately equivalent to 1.070 mg/L of ascorbic acid (Figure 10). The iron chelation assay was intended to help assess the ability of (+)-catechin to chelate metal ions preventing them from initiating radical chain reactions. However, high variability between measurements of both the samples and the standard curve solutions prevented this. Multiple attempts were made to improve the process, this included: acid washing glassware to remove interfering metal ions, using milli-O filtered DI water, creating new buffer and reagent solutions, and increasing the number of measurements taken per sample per run, and increasing the number of runs. Despite this, the highest R^2 value that could be obtained was 0.9216 which is not reliable. Regardless, all the (+)-catechin samples that were run, resulted in absorbances above the measured EDTA curve range (Figure 11). This indicates that (+)-catechin does not function as an iron chelator at the concentrations that were tested. While iron chelation may not be possible with this compound, chelation of other metals known to initiate radical reactions, such as copper and zinc, should still be investigated.

As previously mentioned, increased absorbance of radicals formed during the oxidation of (+)-catechin overshadowed the decay in absorbance that was expected from the PGR probe.

Spartan 2.0 was used to find the identity and predict the UV-Vis absorbance spectra of these radicals (Figures 12A through 12J). The four radicals that were identified were compared to the optimal wavelengths needed to measure PGR absorbance and no optimal wavelength was found that would eliminate the issue. In the end, the ORAC assay had to be eliminated from further use in this study. The total radical trapping antioxidant parameter (TRAP) assay is also commonly used to monitor this type of antioxidant potential. Given the issue with the ORAC assay, it may prove useful in future studies.

Cell Assays

The original research plan was to use flow cytometry to measure the different reactions of a fluorescent probe (CellROX Orange) when combined with an oxidant, (+)-catechin, a blank, a positive or a negative control. With the use of the SYTOX Red dye, the assay would have also given a basic understanding of how the addition of (+)-catechin effects the amount of cell death. It was expected that the addition of the (+)-catechin would inhibit cytoplasmic oxidation, and in turn result in less fluorescence (inhibited cytoplasmic oxidation) and apoptosis compared to the positive control. During the first run of the flow cytometry experiments the instrument suffered a critical error that could not be rectified. Data was collected for the negative control (NAC), positive control, a blank (water), and for the 50 μM (+)-catechin sample but not for the 20 or 5 μM (+)-catechin samples, and no replicates could be completed. The fluorescence of the 50 μM (+)-catechin was higher than the blank and the antioxidant control but was less than and the positive control. There could possibly be a weak relationship between the addition of (+)-catechin (at this concentration) and the inhibition of cytoplasmic oxidation but this could not be shown with statistical significance. SYTOX red results were inconclusive as (+)-catechin, and both controls had very similar results (Figures 13A through 13C).

The same flow cytometry dye kit was used to stain cells and measure the fluorescence using a 96-well plate reader. This data returned conflicting results. Again, the 50 μM (+)-catechin samples showed decreased fluorescence compared to the positive control and greater fluorescence compared to the negative control. However, as opposed to the flow cytometry data, the fluorescence was less than the blank (Figure 14). The 5 and 20 μM samples did not follow this trend. The fluorescence for both samples was greater than all the control and blank samples (Figure 14). While these measurements are still not statistically significant, they do reiterate the possible relationship between the high dose (+)-catechin and inhibited cytoplasmic oxidation that needs further investigation. There could also be a possible dose response wherein (+)-catechin acts at a pro-oxidant at low concentrations and an antioxidant once a concentration threshold is reached.

To better understand the suggested relationships between (+)-catechin and cytoplasmic oxidation additional experiments with statistical significance are needed. Future studies should focus on flow cytometry analysis and possibly assess more than just three concentrations of (+)-catechin. The use of multiple cell populations in these experiments would be ideal since living cells can exhibit a high level of variability.

Summary

Dioscorea bulbifera contains many different phenolic compounds based on this study and others like it (Liu et al 2011; Ghosh et al 2015). This study found that (+)-catechin is one of the major chemical components in the bulbils of this species (Champagne et al 2011). It also determined that (+)-catechin would likely act as an electron donating antioxidant but be a very poor iron chelator based on the observed FRAP and iron chelation assay results. Hydrogen transfer antioxidant potential could not be measured but is worth investigating further to get a broader understanding of this compound. Lastly, at higher concentrations (+)-catechin may act as an

antioxidant but at lower concentrations it appeared to increase oxidation within the cytoplasm of human neutrophils.

In reference to the hypotheses of this study, the above findings were inconclusive. The first hypothesis was that (+)-catechin would need to be minimally cytotoxic at low concentrations. Without the use of the flow cytometer to measure the SYTOX Red fluorescence, the cytotoxicity could not be assessed. The second hypothesis was that (+)-catechin would be able to inhibit cytoplasmic oxidation. While it appears that this may be possible, it is not known if this observation is a result of (+)-catechin acting within the cell, or through the neutralization of TBHP within the growth media outside the cell. Hypothesis three stated that because of the numerous hydroxyl groups, (+)-catechin was expected to act as an antioxidant via hydrogen transfer rather than electron transfer or metal chelation. Without the ORAC data, the hydrogen transfer mechanism could not be assessed. Contrary to the predicted outcome, (+)-catechin showed potential for electron transfer. In support of hypothesis three, (+)-catechin was shown to be a very poor iron chelator. Considered together the conclusions of these hypotheses were supposed to support the use of (+)-catechin as an antioxidant with possible medicinal properties. This conclusion was ultimately not supported. Rather, much more investigation is needed before such a statement can be made.

REFERENCES

Amorati R, Valgimigli L (2015) Advantages and limitations of common testing methods for antioxidants. Free Radical Research 49:633-649. doi: 10.3109/10715762.2014.996146.

Bast A, Haenen, Guido R M (2013) Ten misconceptions about antioxidants. Trends in Pharmacological Sciences 34:430-436. doi: 10.1016/j.tips.2013.05.010.

Bender C, Graziano S (2015) Evaluation of the antioxidant activity of foods in human cells: Integrated study of biologically active antioxidants from Camellia sinensis. NutraFoods 14:79-85.

Bryan N (2018) Functional Nitric Oxide Nutrition to Combat Cardiovascular Disease. Current Atherosclerosis Reports 20:1-12. doi: 10.1007/s11883-018-0723-0.

Carocho M, Morales P, Ferreira, Isabel C F R (2018) Antioxidants: Reviewing the chemistry, food applications, legislation and role as preservatives. Trends in food science & technology 71:107-120.

Cascão R, Rosário HS, Souto-Carneiro MM, Fonseca JE (2010) Neutrophils in rheumatoid arthritis: More than simple final effectors. Autoimmunity Reviews 9:531-535. doi: 10.1016/j.autrev.2009.12.013.

Cavnar PJ, Berhtier E, Beebe DJ, Huttenlocher A (2011) Hax1 regulates neutrophil adhesion and motility through RhoA. Journal of Cell Biology 193:465-473.

Champagne A, Hilbert G, Legendre L, Lebot V (2011) Diversity of anthocyanins and other phenolic compounds among tropical root crops from Vanuatu, South Pacific. Journal of Food Composition and Analysis 24:315-325. doi: 10.1016/j.jfca.2010.12.004.

Chen S, Li M, Zheng G, Wang T, Lin J, Wang S, Wang X, Chao Q, Cao S, Yang Z, Yu X (2018) Metabolite Profiling of 14 Wuyi Rock Tea Cultivars Using UPLC-QTOF MS and UPLC-QqQ MS Combined with Chemometrics. Molecules 23:1-18.

Comert ED, Gokmen V (2018) Evolution of food antioxidants as a core topic of food science for a century. Food research international 105:93.

de Blas E, Estañ MC, del Carmen Gómez de Frutos, María, Ramos J, del Carmen Boyano-Adánez M, Aller P (2016) Selected polyphenols potentiate the apoptotic efficacy of glycolytic inhibitors in human acute myeloid leukemia cell lines. Regulation by protein kinase activities. Cancer Cell International 16:1-16. doi: 10.1186/s12935-016-0345-y.

Dewick PA (2009) Medicinal Natural Products: A Biosynthetic Approach. John Wiley & Sons Ltd., West Sussex, United Kingdom.

Fang Z, Wu D, Yü D, Ye X, Liu D, Chen J (2011) Phenolic compounds in Chinese purple yam and changes during vacuum frying. Food Chemistry 128:943-948. doi: 10.1016/j.foodchem.2011.03.123.

Fattori V, Amaral FA, Verri WA (2016) Neutrophils and arthritis: Role in disease and pharmacological perspectives. Pharmacological Research 112:84-98. doi: 10.1016/j.phrs.2016.01.027.

García-Salas P, Gómez-Caravaca AM, Morales-Soto A, Segura-Carretero A, Fernández-Gutiérrez A (2015) Identification and quantification of phenolic and other polar compounds in the edible part of Annona cherimola and its by-products by HPLC-DAD-ESI-QTOF-MS. Food Research International 78:246-257. doi: 10.1016/j.foodres.2015.10.002.

Ghosh S, More P, Derle A, Patil AB, Markad P, Asok A, Kumbhar N, Shaikh ML, Ramanamurthy B, Shinde VS, Dhavale DD, Chopade BA (2014) Diosgenin from Dioscorea bulbifera: novel hit for treatment of type II diabetes mellitus with inhibitory activity against α-amylase and α-glucosidase. PloS one 9:1-9. doi: 10.1371/journal.pone.0106039.

Ghosh S, Parihar VS, Dhavale DD, Chopade BA (2015) Phytochemistry and Therapeutic Potential of Medicinal Plant Dioscorea bulbifera. Medicinal Chemistry 5:160-172.

Gutteridge JMC, Halliwell B (2010) Antioxidants: Molecules, medicines, and myths. Biochemical and Biophysical Research Communications 393:561-564. doi: 10.1016/j.bbrc.2010.02.071.

Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry 53:1841-1856.

Jiang J, Xiong YI (2016) Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. Meat science 120:107-117.

Khonkarn R, Okonogi S, Ampasavate C, Anuchapreeda S (2010) Investigation of fruit peel extracts as sources for compounds with antioxidant and antiproliferative activities against human cell lines. Food and Chemical Toxicology 48:2122-2129.

Knoefler D, Leichert LIO, Thamsen M, Cremers CM, Reichmann D, Gray MJ, Wholey W, Jakob U (2014) About the dangers, costs, and benefits of living an aerobic lifestyle. Redox Regulation in Health and Disease 42:917-921.

Kolniak-Ostek J, Oszmiański J (2015) Characterization of phenolic compounds in different anatomical pear (Pyrus communis L.) parts by ultra-performance liquid chromatography photodiode detector-quadrupole/time of flight-mass spectrometry (UPLC-PDA-Q/TOF-MS). International Journal of Mass Spectrometry 392:154-163. doi: 10.1016/j.ijms.2015.10.004.

Kumar S, Kumar Parida A, Kumar Jena P (2013) Ethno-Medico-Biology of Bān-Aālu (Dioscorea species): A neglected tuber crops of Odisha, India. International Journal of Pharmacy & Life Sciences 4:3143-3150.

Langeland KA, Enloe SF (2001) Natural Area Weeds: Air Potato (Dioscorea bulbifera) 2018.

Li H, Horke S, Förstermann U (2014) Vascular oxidative stress, nitric oxide and atherosclerosis. Atherosclerosis 237:208-219. doi: 10.1016/j.atherosclerosis.2014.09.001.

Li Z, Guo H, Xu W, Ge J, Li X, Alimu M, He D (2016) Rapid Identification of Flavonoid Constituents Directly from PTP1B Inhibitive Extract of Raspberry (Rubus idaeus L.) Leaves by HPLC–ESI–QTOF–MS-MS. Journal of Chromatographic Science 54:805-810. doi: 10.1093/chromsci/bmw016.

Liu H, Tsim KWK, Chou G, Wang J, Ji L, Wang Z (2011) Phenolic Compounds from the Rhizomes of Dioscorea bulbifera. Chemistry and Biodiversity 8:2110-2116.

López-Alarcón C, Denicola A (2013) Review: Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. Analytica Chimica Acta 763:1-10.

López-Alarcón C, Lissi E (2006) A novel and simple ORAC methodology based on the interaction of Pyrogallol Red with peroxyl radicals. Free Radical Research 40:979-985. doi:10.1080/10715760500481233.

MassBank of North America (2018) A public repository for sharing mass spectral data for life sciences. http://mona.fiehnlab.ucdavis.edu/.

Martins N, Barros L, Ferreira, Isabel C F R (2016) In vivo antioxidant activity of phenolic compounds: Facts and gaps. Trends in Food Science & Technology 48:1-12.

Mikulic-Petkovsek M, Ivancic A, Todorovic B, Veberic R, Stampar F (2015) Fruit phenolic composition of different elderberry species and hybrids. Journal of Food Science 80:C2190. doi: 10.1111/1750-3840. 13008.

Mobasheri A, Batt M (2016) An update on the pathophysiology of osteoarthritis. Annals of Physical and Rehabilitation Medicine 59:333-339.

Negi PS (2012) Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology 156:7-17. doi: 10.1016/j.ijfoodmicro.2012.03.006.

Niki E (2010) Assessment of Antioxidant Capacity in vitro and in vivo. Free Radical Biology and Medicine 49:503-515. doi: 10.1016/j.freeradbiomed.2010.04.016.

Odobasic D, Kitching AR, Holdsworth SR (2016) Neutrophil-Mediated Regulation of Innate and Adaptive Immunity: The Role of Myeloperoxidase. Journal of Immunology Research 2016:1-11.

Oroian M, Escriche I (2015) Antioxidants: Characterization, natural sources, extraction and analysis. Food Research International 74:10-36. doi: 10.1016/j.foodres.2015.04.018.

Ortiz R, Antilen M, Speisky H, Aliaga ME, López-Alarcón C (2011) Analytical paramters of the microplate-based ORAC-pyrogallol red assay. Journal of AOAC International 94:1562-1566. doi: 10.5740/jaoacint. 11-033.

Phaniendra A, Jestadi DB, Periyasamy L (2015) Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian Journal of Clinical Biochemistry 30:11-26.

Pinegin B, Vorobjeva N, Pinegin V (2015) Neutrophil extracellular traps and their role in the development of chronic inflammation and autoimmunity. Autoimmunity reviews 14:633.

Pisoschi AM, Pop A (2015) The role of antioxidants in the chemistry of oxidative stress: A review. European journal of medicinal chemistry 97:55-74. doi:10.1016/j.ejmech.2015.04.040.

Poprac P, Jomova K, Simunkova M, Kollar V, Rhodes CJ, Valko M (2017) Review: Targeting Free Radicals in Oxidative Stress-Related Human Diseases. Trends in pharmacological sciences 38:592-607.

Prior RL (2015) Oxygen radical absorbance capacity (ORAC): New horizons in relating dietary antioxidants/bioactives and health benefits. Journal of Functional Foods 18:797-810. doi: 10.1016/j.jff.2014.12.018.

Pruchniak MP, Arazna M, Demkow U (2013) Life of neutrophil: from stem cell to neutrophil extracellular trap. Respiratory Physiology & Neurobiology 187:68-73. doi: 10.1016/j.resp.2013.02.023.

Pulido R, Bravo L, Saura-Calixto FD (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry 48:3396-3402.

Rajendran P, Nandakumar N, Rengarajan T, Palaniswami R, Gnanadhas EN, Lakshminarasaiah U, Gopas J, Nishigaki I (2014) Antioxidants and human diseases. Clinica Chimica Acta 436:332-347. doi: //dx.doi.org/10.1016/j.cca.2014.06.004.

Rothwell JA, Perez-Jimenez J, Neveu V, Medina-Remón A, M'hiri N, García-Lobato P, Manach C, Knox C, Eisner R, Wishart DS, Scalbert A (2013) Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content. Database: the journal of biological databases and curation 2013:bat070. doi: 10.1093/database/bat070.

Sarangarajan R, Meera S, Rukkumani R, Sankar P, Anuradha G (2017) Antioxidants: Friend or foe? Asian Pacific Journal of Tropical Medicine 10:1111-1116.

Shahidi F, Ambigaipalan P (2015) Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. Journal of Functional Foods 18:820-897. doi: 10.1016/j.jff.2015.06.018.

Soehnlein O, Steffens S, Hidalgo A, Weber C (2017) Neutrophils as protagonists and targets in chronic inflammation. Nature Reviews. Immunology 17:248-261. doi: 10.1038/nri.2017.10.

Souza de Silva G, Canuto KM, Ribeiro PRV, Sousa de Brito E, Nascimento MM, Zocolo GJ, Coutinho JP, Mota de Jesus R (2017) Chemical profiling of guarana seeds (paullinia cupana) from different geographical origins using UPLC-QTOF-MS combined with chemometrics. Food Research Journal 100:700-709.

Veselinovic M, Barudzic N, Vuletic M, Zivkovic V, Tomic-Lucic A, Djuric D, Jakovljevic V (2014) Oxidative stress in rheumatoid arthritis patients: relationship to diseases activity. Molecular & Cellular Biochemistry 391:225-232.

Vrolijk MF, Opperhuizen A, Jansen, Eugene H J M, Godschalk RW, Van Schooten FJ, Bast A, Haenen, Guido R M (2015) The shifting perception on antioxidants: the case of vitamin E and B-carotene. Redox Biology 4:272-278.

Wang J, Arase H (2014) Regulation of immune responses by neutrophils. Annals of the New York Academy of Sciences 1319:66-81. doi: 10.1111/nyas.12445.

Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, Sayeeda Z, Lo E, Assempour N, Berjanskii M, Singhal S, Arndt D, Liang Y, Badran H, Grant J, Serra-Cayuela A, Liu Y, Mandal R, Neveu V, Pon A, Knox C, Wilson M, Manach C, Scalbert A (2018) HMDB 4.0: the human metabolome database for 2018. Nucleic acids research 46:D617. doi: 10.1093/nar/gkx1089.

Yang J, Qian D, Guo J, Jiang S, Shang E, Duan J, Xu J (2013) Identification of the major metabolites of hyperoside produced by the human intestinal bacteria using the ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry. Journal of ethnopharmacology 147:174-179. doi: 10.1016/j.jep.2013.02.029.

Zhang H, Tsao R (2016) Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. Current Opinion in Food Science 8:33-42. doi:10.1016/j.cofs.2016.02.002.

Zhu J, Yi X, Zhang J, Chen S, Wu Y (2017) Chemical profiling and antioxidant evaluation of Yangxinshi tablet by HPLC-ESI-Q-TOF-MS/MS combined with DPPH assay. Journal of Chromatography B 1060:262-271.