IN VIVO MODULATION OF REDOX AND NITRIC OXIDE SIGNALING BY LAMIACEAE PHYTOCHEMICALS

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ABSTRACT

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Cells are constantly exposed to free radicals as part of normal metabolic processes. However, in certain conditions the homeostatic balance that exists between free radicals and cellular antıoxidants can be altered, resulting in oxidative stress. In this state, excess free radicals can damage surrounding macromolecules and subsequently inhibit normal cell function which can lead to the progression of many chronic diseases. Plant secondary metabolites are biologically active molecules known to effectively manage adverse health conditions associated with oxidative stress. In fact, epidemiological data has shown that diets rich in plant foods are inversely related to risk for chronic disease. Herbs and spices generally are concentrated sources of a variety of

plant secondary metabolites including phenolic compounds, carotenoids, saponins, and alkaloids. These phytochemicals have chemical structures and electrochemical properties that are capable of modulating the intracellular redox environment by stabilizing free radicals and by increasing the activity and/or expression of important antioxidant enzymes. The Lamiaceae herb family is large group containing approximately 3200 species grown worldwide for medicinal and culinary purposes. Research has previously shown these herbs to have antibacterial, antiviral, antioxidant, and anti-cancer properties which are largely contributed to their phytochemical profiles. The recent evaluations of these plant's secondary metabolites and their biological functions have importance as therapeutic agents for various health implications However, the mechanism of action by which this occurs in vivo is not very well understood. Therefore, the objectives of this study were to determine the biochemical, physiological, and molecular effects of plant secondary metabolites from Lamiaceae herbs on redox and nitric oxide signaling in vivo in *Lumbricus terrestris*. We have adapated and developed *L. terrestris* as a model system to study oxidative stress (Hutton et al., 2009). In the current study, the effect of dietary Lamiaceae herbs on modulating redox/nitric oxide (NO) signaling and sperm quality in the oxidation prone environment of seminal vesicles was determined. Animals fed ad libitum on *Lumbricus* growth medium (LGM) supplemented with 0% (control), 0.1% or 0.5% (w/v) of different herbs. Additionally, the effects of different combinations of Lamiaceae herbs on modulating redox/nitric oxide signaling in *L. terrestris* were conducted. In this objective, the standard LGM was supplemented with two different Lamiaceae herbs, each at a concentration of 0.05% (w/v). Also, the modulatory effects of Lamiaceae herbs on a peroxide induced oxidative stress were studied. The seminal

vesicles of the animal were dissected out on day 2 and day 6, and gently disrupted. Levels of malondialdehyde (MDA), DNA fragmentation (DNAF), glutathione (GSH), nitrates/nitrites (NOx), superoxide dismutase (SOD), catalase (CAT) were determined using standard assays. Sperm maturity and deformation (DFO) was quantified microscopically. Data analysis suggests modulation of redox response via protein kinase C, ARE-Nrf2 and AP-2 mediated expression of SOD, glutathione peroxidase and Nitric oxide synthase. Additionally, to understand the mechanism of redox modulation by Lamiaceae herbs we used transgenic strains of *Caenorhabditis elegans* with transcriptional reporter (GFP) constructs of relevant genes. The *C. elegans* model confirmed the observations in *L. terrestris*. All herbs had differential effects on expression of different genes considered for this study, perhaps due to different bioactive constituents.

CHAPTER I

THE PHYSIOLOGICAL EFFECTS OF DIFFERENT LAMIACEAE HERBS ON REDOX AND NITRIC OXIDE SIGNALING IN SEMINAL VESICLES OF *LUMBRICUS TERRESTRIS*

Oxidative Stress Mediated Pathogenesis of Disease

As early eukaryotic organisms evolved, they developed protective mechanisms against molecular oxygen and its highly reactive metabolites, allowing oxygen to be incorporated into various biochemical reactions and cellular processes (Raymond and Segre, 2006; Ott et al., 2007). Molecular oxygen has most importantly enabled organisms to efficiently produce ATP through its role as the ultimate acceptor of electrons in the electron transport chain. However, the reduction of molecular oxygen is inevitably associated with the formation of reactive oxygen species (ROS) via enzymatic or metal catlyzed reactions such as in beta oxidation, electron transport chain, or the fenton reaction (Gershman et al., 1954; Harman, 1956; Rae et al., 1999; Valko et al., 2005). The term reactive oxygen species includes free radicals, non-radical molecules, reactive nitrogen species, and chlorine species (Karıhtala and Soini, 2007; Valko et al., 2007). Free radicals are characterized by having one or more unpaired electrons and possess unique configurations and chemical structures that affect its ability to diffuse through cells and react with macromolecules (Halliwell and Gutteridge, 1989; Tsukahara, 2007).

A few examples of ROS and free radicals include the superoxide anion, hydrogen peroxide, the hydroxyl radical, peroxynitrite radical, hypochlorous acid, and nitrogen dioxide (Cadenas and Sies, 1998; Karihtala and Soini, 2007; Lieberman and Marks, 2009). The superoxide anion is considered the "primary" ROS as it has the ability to generate other reactive species (Valko et al., 2007). This free radical can be generated by the one electron reduction of molecular oxygen in the electron transport chain or by enzymes such as NADPH oxidase (Babior, 1999; Cadenas and Davies, 2000; Pervaiz and Clement, 2007). Superoxide is able to release iron from enzymes containing iron-sulfur centers and promote iron catalyzed Fenton reaction which leads to the eventual production of the hydroxyl radical (Liochev and Fridovich, 1994). ROS can also be produced when organisms are exposed to exogenous factors such as ionizing radiation and xenobiotics (Freeman and Crapo, 1982; Finkel and Holbrook, 2000; Nguyen et al., 2004). Being unstable and reactive, free radicals participate in spontaneous electron abstraction from surrounding macromolecules causing oxidative modification to lipids, proteins, and nucleic acids which eventually alter normal cell physiology (Halliwell and Gutteridge, 1989; Siems et al., 1995).

In all oxygen metabolizing organisms there exists a precise balance between prooxidants generated during cellular respiration and the antioxidant defense systems (Seifried et al., 2007). Evidence from many studies suggest that ROS are not only harmful by-products of cellular metabolism but also essential in many biological processes (Thannickal and Fanburg, 2000). For example, it is now well known that cells of the immune system produce superoxide anions to kill invading pathogens (Robinson, 2008). These molecules are not only important in the immune response, but are also

capable of activating signaling pathways as was shown in the the activation of mitogen-activated protein kinase (MAPK) by hydrogen peroxide (Finkel, 1998). The modulation of important cellular pathways can induce apoptosis or necrosis and alter gene expression which directly affects cell survival (Hancock et al., 2001). However, during overproduction of reactive oxygen species and/or suboptimal functioning antioxidant defenses can lead to a metastable condition characterized as oxidative stress (Azzi, 2007). If oxidative stress persists, excess ROS can damage biomolecules that play critical roles in cell survival and these include DNA, RNA, lipids, and proteins (Hunt et al., 1998). Oxidative stress can eventually disrupt normal cellular metabolism, alter cell signaling pathways, effect gene expression, and cell survival (Monteiro and Stern, 1996; Sun and Oberly, 1996; Tan et al., 1998). Oxidative stress is now implicated with the pathogenesis of many diseases such as diabetes, cancer, and cardiovascular disease (Jakus, 2000; Droge, 2002; Madamanchi et al., 2005; Lin and Beal, 2006; Federico et al., 2007; Tremellen, 2008).

Antioxidant Defense Systems

Living organisms have evolved many antioxidant and protective mechanisms against oxygen and its highly reactive metabolites (Cadenas, 1997). These include the endogenous enzymes superoxide dismutase, catalase, and glutathione peroxidase that can metabolize free radicals. Additionally, antioxidant molecules such as glutathione, selenium, coenzyme Q, and vitamins C and E have high electron donating potentials and they also participate in the removal of ROS (Halliwell, 1994; Mates and Sanchez-Jimenez, 1999; Mates, 2000).

Superoxide dismutase (SOD) was discovered in the 1960s by McCord and Fridovich in bovine erythrocytes where they described its functions as an enzyme which catalyzes the dismutation of superoxide radicals ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$) (McCord and Fridovich, 1969). Since the discovery of SOD, there are four isoforms of the enzyme which have been identified and they include Ni-SOD (cytoplasmic), Mn-SOD (mitochondrial), Cu/Zn-SOD (cytoplasmic), and EC-SOD (extracellular) (Mates and Sanchez-Jimenez, 1999). The product of the dismutation of two superoxide anions carried out by SOD is the ROS, hydrogen peroxide, therefore biologically this enzyme is often coupled with antioxidants that remove hydrogen peroxide such as catalse and glutathione peroxidase.

Catalase is capable of enzymatically removing hydrogen peroxide to form water and molecular oxygen (Agar et al., 1986; Mates et al., 1999). This enzyme is distributed in all tissues in most species, and it helps prevent cell damage and the further propagation of free radicals (Harris, 1992). Hydrogen peroxide, if not removed from a system, can lead to the formation of the extremely reactive hydroxyl radical through the Fenton reaction or Haber-Weiss reaction (Cohen and Heikkila, 1974). Glutathione peroxidase is another enzyme capable of catalytically removing hydrogen peroxide and other hydroperoxides using glutathione (Ladenstein et al., 1979). There are at least eight known isoforms of this enzyme distributed in various tissues of organisms (Mates and Jimenez-Sanchez, 1999).

The major antioxidant molecule, glutathione (GSH), is a tripeptide that is generated within the cytosol via glutamate-cystein ligase and glutathione synthetase (Valko et al., 2007). This molecule has several important functions in the cell which

include it acting as a cofactor for antioxidant enzymes, an aide for amino acid transfer through plasma membranes, a scavenger of free radicals, and a regenerator of oxidized vitamins C and E (Masella et al., 2005).

Together these antioxidant enzymes and molecules maintain a homeostatic balance by neutralizing the constant flux of radicals generated through cellular metabolic processes and exogenous sources (Droge, 2002).

Phytochemicals

There is growing evidence from epidemiological studies that indicate diets rich in fruits, vegetables, whole grains, and nuts are associated with decreased risks for the development of cardiovascular disease, cancer, diabetes, and other chronic diseases (Hu, 2003; Key et al., 2004; Martinez-Gonzalez et al., 2008). Plants provide us with nutrients necessary for normal cell functions but they also contain bioactive non-nutrients which may provide desirable health benefits beyond regular nutrition (Liu, 2003). These bioactive chemicals or phytochemicals are synthesized as adaptations to the environment in which the plant is grown. They protect the plant from stressors such as herbivores, insects, parasites and pathogens, and high energy radiation. In addition these can also protect against drought and cold (Pourcel and Grotewold, 2009). Thousands of bioactive compounds have been identified in plants. They vary significantly among species, varieties, and cultivars, however broad classes of major phytochemicals have been established and they include phenolics, terpenoids, and alkaloids or nitrogen-derived compounds (Manach et al., 2009).

Phenolic phytochemicals are one of the most widely distributed secondary metabolites occurring among plants with over 4,000 phenolic compounds identified

(King and Young, 1999). These molecules are distinguished by the presence of aromatic rings with varied numbers of hydroxyl groups attached, and they are further divided into subcategories based on the number of phenol rings in the molecule and structural elements bound to these rings (Manach et al., 2004) (Table 1). Flavonoids are low molecular weight phenolic compounds characterized by two or more aromatic rings bound by a carbon bridge with variable amounts of hydroxyl groups attached (Beecher, 2003). This class of phytochemicals is most ubiquitous in nature and can be further divided into subcategories based on chemical structures and substitutions (glycosylation, sulphation, methylation) which include flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (Cook and Samman, 1996). Phenolic acids are another of the major categories of phenolic compounds found in many plants. These molecules have the simplest chemical structure with a single phenolic ring and most phenolic acids are derivatives of benzoic and cinnamic acid (Natella et al., 1999). Phenolic acids rarely freely exist in nature but are usually bound to other molecules, and they have diverse functions in the plant which include roles in photosynthesis, enzyme activity, and nutrient uptake (Robbins, 2003). Lignans are another category of phenolic phytochemicals identified as compounds containing a 1,4 diarylbutane structure and they are found in plants such as cereals, grains, fruits, and vegetables (Cassidy et al., 2000). These compounds are metabolized by gut microflora into molecules such as enterolactone and enterdiol of which are known to have estrogen-like effects in the body and are of great interest to researchers for their impact on human health (Manach et al., 2004). The fourth major category of phenolic phytochemicals are stilbenes. These molecules are 1,2-diarylethenes and are found in a wide range of plants in roots, barks, and leaves (Cassidy

et al., 2000). Stilbenes are not widespread in food plants, however they occur in minimal amounts in grapes and peanuts (Scalbert and Williamson, 2000). Resveratrol is the most commonly studied stilbene as it is proposed to have anticarcinogenic, antioxidant, and anti-inflammatory properties (Savouret and Quesne, 2002). Another widely distributed polyphenolic compound distributed in many fruits, vegetables, seeds, grains, and beverages are tannins. Structurally these compounds are bulky, ranging in molecular weights between 500 and 3000, and have been divided into hydrolysable tannins and proanthocyanidins or condensed tannins (Santos-Buelga and Scalbert, 2000). The name for this compound is derived from its ability to precipitate proteins. Oak, a commonly used plant used in tanning the hides of animals, is a rich source of tannins that facilitates in this process (Hagerman, 1998). *In vitro* evidence has indicated that these polyphenolic compounds are 15-30 times more effective at quenching radicals than structurally simple phenolics (Hagerman et al., 1998). Catechins are another class of flavonoids that investigators have shown to have several health benefits associated upon consumption of these compounds. Rich sources of these polphenolic compounds are teas, particularly green tea, which has several catechins such as catechin gallate, epigallocatechin, and epigallocatechin gallate (Valcic et al., 1999). Among all catechins, Epigallocatechin gallate (EGCG) has been extensively studied and results have indicated antioxidant properties, anticarcinogenic properties, and effects associated with alterations in lipid metabolism (Jung et al., 2001; Lu et al., 2002; Raederstorff et al., 2003).

Table 1: Classes of Phenolic Phytochemicals and Food Sources

Class	Subclass	Phytochemicals	Food Source
Flavonoids			
	Flavonols	quercitin, kaempferol,	onions, apples, kale, broccoli, tea, cranberry
	Flavones	apigenin, tangeritin, wogonin	celery, olives, tangerines
	Isoflavones	genestein, daidzein	soy, peanuts, chick peas
	Flavanones	hesperidin, hesperetin, naringin	oranges, limes, lemons,
	Flavanols	catechin, epicatechin	tea, pears, apples, wine
Phenolic Acids			
	Hydroxycinnamic acid	caffeic, ferulic, chlorogenic	rosemary, sage, oregano, grapefruit, potato
	Hydroxybenzoic acid	ellagic acid, gallic acid	raspberry, strawberry, grapes
Tannins			
	Condensed	catechin, epigallocatechin	walnuts, teas, wines, chocolate
	Hydrolyzable	punicalagans	pomegranate

Phytochemicals from Lamiaceae herbs

Historically herbs and spices have been of great interest to many people, and in earlier times sailors would travel to various part of the world in search of these valuable plants. The trade industry for herbs and spices became a major part of the economy for countries such as India and China. These plants were not only revered for their flavor imparted to culinary dishes, but people also recognized the medicinal properties (Agqarwal and Shishodia, 2004). Currently, several varieties of herbs and spices are grown worldwide and only recently have researchers begun to systematically investigate the functional properties associated with natural products. One highly researched herb family, the Lamiaceae family, is large group containing approximately 3200 species grown worldwide for medicinal and culinary purposes (Mimica-Dukic and Bozin, 2008). Medicinally these plants have been used by various cultures as a cholerectics, antiseptics,

GI disorder treatments, hypoglycemic drugs, anti-inflammatories, antibacterials, and diuretics (Shetty, 1997). The recent evaluations of these plant's secondary metabolites and their biological functions have importance as therapeutic agents for various health implications.

The leaves of sage are frequently used in culinary preparations, but it has only recently been evaluated for its effects on human health. Traditionally this plant has been used to treat ailments such as colds, abdominal pain, stomach ulcers, headaches, and rheumatism (Rivera et al., 1994; Muhtasib et al., 2000). The medicinal usage of this plant in traditional medicine has prompted several researchers to investigate the phytochemical composition of this genus as well as some of the functional properties associated with these molecules. The chemical components of sage are very complex and include diterpenes, triterpenes, and several other phenolic compounds (Wang et al., 1998). It has been indicated that most of the secondary metabolites in sage are phenolic compounds such as rosmarinic acid, caffeic acid, apigenin, hispidulin, and cirsimaritin (Areias et al., 2000). In one particular study using water extracts of four different Lamiaceae herbs, sage was shown to have the highest phenolic content which was also correlated with a high free radical reductive capacity (Dorman et al , 2003). The antioxidant properties of sage have been investigated in several studies. Treatment with sage has shown to prevent LDL oxidation, increase glutathione levels, and reduce DNA fragmentation (Triantaphyllou et al., 2001; Lima et al., 2005; Iuvone et al., 2006; Aherne et al., 2007). Rosmarinic acid, a predominant phenolic compound in sage, has been shown to have anti-inflammatory, anti-viral, and antioxidant effects thereby giving it the potential to function as pharmacological agent (Shetty, 1997). It fact sage has been added to products

such as toothpaste where it has been shown to reduce plaque growth, and it was recently used as a topical agent where it was shown to have anti-inflammatory properties greater than that of a common non-steriodal anti-inflammatory drug (NSAID) (Bricevic et al., 2001).

Basil is another Lamiaceae herb primarily used for culinary purposes and it is grown in sub tropical regions all over the world with nearly 150 different species. However, different cultures have traditionally used basil as a means to treat warts, constipation, kidney malfunctions, bronchitis, and sore throats (Vieira and Simon, 2000; Javanmardi et al., 2002). Evaluations of secondary metabolites in basil have revealed an array of phytochemicals that include monoterpenes, sesquiterpenes, and aromatic compounds (Lee et al., 2005). Ethanolic extracts and preparation of essential oils from a variety of basil species were shown to have high antioxidant properties *in vitro* (Juliani and Simon, 2002). Ursolic acid, a triperpenoid found in basil, was able to suppress transcription factor nuclear factor-kappa B (NF-κB) mediated signaling by inhibiting I kappa kinase (IKK) which lead to the suppression of many inflammatory mediators (Aggarwal and Shishodia, 2004).

The genus, Thyme, contains hundreds of known species and it is extensively cultivated for its use as a culinary and medicinal herb (Hanrahan and Odle, 2005). Like most other Lamiaceae herbs, thyme is an abundant source of phytochemicals which have antioxidant properties (Miura and Nakatani, 1989). The essential oils from thyme, thymol and carvacrol, have been shown to inhibit the growth of common pathogens such as *Salmonella typhimurium* and *Staphlococcus aureus* (Juven et al., 1993). The oils from thyme have also shown anti-aging effects in a mouse model (Youdim and Deans, 2000).

Supplementation with thyme oil was shown to reduce age related declines in antioxidant enzyme activity and it was also able to maintain appropriate concentrations of fatty acids in the brains of aging mice (Youdim and Deans, 2000). The Mediterranean diet has associated with lower incidences of cardiovascular disease, and it is thought to be partly derived from foods rich in phenolic phytochemicals (Caluccio et al., 2003). Thyme is an herb frequently used in culinary preparations of this area, and it was recently shown that extracts of wild thyme were able to increase nitric oxide production in endothelial cells important for regulation of blood pressure (Grande et al., 2004). This herb has thrombic activity as indicated by its ability to inhibit platelet aggregation when exposed to collagen. This property associated with thyme is implicated with lowering of cardiovascular disease (CVD) risks as thrombosis and arteriosclerosis are strongly associated with platelet aggregation (Okazaki et al., 2002).

Oregano is native to the regions surrounding the Mediterranean, and became extremely popular in North America following World War II when soldiers developed a taste for Italian culinary dishes flavored with this herb (Paradise, 2005). This plant has been used in traditional medicine for healing wounds, headaches, cold symptoms, and insect bites (Paradise, 2005). Recently, extracts from oregano have been shown to have antibacterial properties. In one study, oregano extracts were able to inhibit *Helicobacter pylori* growth and also able to inhibit urease activity. This enzyme produces alkaline products that allows *Helicobacter pylori* to grow in acidic environments such as the stomach (Lin et al., 2005). Oregano also has antioxidant properties associated with the abundant phenolic phytochemicals. Extracts of oregano reduced oxidative damages

induced by hydrogen peroxide through its ability to quench free radicals and induce an antioxidant response in porcine muscle tissue (Randhir et al., 2005).

Rosemary has been studied extensively in recent years. This plant is grown worldwide and like all Lamiceae herbs played a role in traditional folk medicine with applications in hair growth, relieving respiratory problems, and as an analgesic (Al-Sereitia et al., 1999). Scientists have separated and characterized many of the polyphenolic and flavonoid components found in rosemary. These include rosmarinic acid, carnosic acid, rosmanol, eriocitrin, and genkwanin to name a few (Frankel et al., 1996; Del Bano et al., 2004). Specifically ursolic acid and carnosol, abundant in rosemary, inhibited chemically induced skin tumorigenesis in mice (Huang et al., 1994). Carnosol purified from rosemary and rosemary extract also have antiviral properties and inhibited HIV infection in *in vitro* models (Aruoma et al., 1996). Extracts from this herb are added to food products to prevent oxidation, reduce spoilage, and impact health benefits (Ramirez et al., 2004).

Lamiceae herbs are popular worldwide because of their natural antibacterial and antiviral properties which research has shown to be associated with their unique phytochemical makeup. Many of the benefits attributed to rosemary extracts comes from its ability to function as an antioxidant. Most of these herbs are generally regarded as safe (GRAS) and antioxidants from these herbs may someday replace synthetic antioxidants in all food products (Toussaint et al., 2007).

Invertebrate Model for Natural Product Research: Lumbricus terrestris

The earthworms species, *Eisenia fetida* and *Lumbricus terrestris*, are well established models for environmental toxicology studies as they are cost effective, less

controversial in comparison to vertebrate models, and easy to maintain (Goven et al., 1988; Furst et al., 1993; Burch et al., 1999; Massicote et al., 2004). Recently, the earthworm has garnered attention as an established model for soil restoration and immunotoxicity studies (Butt et al., 1993; Reinecke et al., 2002). It has been shown in several toxicology studies that environmental contaminants can induce oxidative stress in earthworm tissues through increased ROS production and decreased antioxidant defenses (Ribera et al., 2001; Lawson and Yu, 2003; Xue et al., 2009). Previous studies have also focused on earthworm reproductive health, particularly regarding sperm morphology and count, as bioindicators of the genotoxic effects of commonly used pesticides (Zang et al., 2000; Bustos-Obregon et al., 2005). Recently our lab has utilized *Lumbricus terrestris* as a model organism. Specifically, to study the biological acitivity of natural products, where we have developed a standardized growth medium and protocols for treatments, extraction of seminal vesicles, muscle tissues, immune cells and to study their functional parameters (Hutton et al., 2009 and Thesis).

Additionally availability of expressed sequence tags (EST) for various genes in the earthworm genome has allowed scientists to use molecular tools to study transcriptional effects of various treatments (Andre et al., 2009; Guo et al., 2009; Sturzenbaum et al., 2009). Microarray technology, nuclear magnetic resonance-based metabolic profiling, and a database dedicated to earthworm genomics are among the recently developed tools that enable researchers to evaluate mechanistic information in this newly established model (Owen et al., 2008; Bundy et al., 2008). In the current study, *Lumbricus terrestris* was used to investigate the effects of plant secondary metabolites from herbs belonging to the Lamiaceae family on biochemical and

physiological responses associated with redox and nitric oxide signaling. The seminal vesicles were the target organ of the current study as it is an effective biomarker of oxidative stress induced toxicities. The extent of oxidative damage was measured through levels of lipid oxidation, morphological deformations in spermatozoa, and through the extent of DNA fragmentation in these tissues. In addition, the antioxidant response in these organisms was measured after treatment with Lamiaceae herbs by evaluating superoxide dismutase activity, catalase activity, and reduced glutathione levels. Similarly, previous studies have also measured antioxidant activity in earthworms used in environmental toxicological research (Saint-Denis et al., 1999; Lawson and Yu, 2003).

Objective of Study

The objective of this study is to determine the effect of different Lamiaceae herbs on redox and NO signaling in seminal vesicles of *Lumbricus terrestris*. Specifically, the effect of Lamiaceae phytochemicals on redox status, antioxidant enzymes (superoxide dismutase, catalase), oxidative damage to DNA, and NO mediated spermatogenesis.

Materials and Methods

Animals: Selection and Treatment

Earthworms of the species *Lumbricus terrestris* were selected based on the presence of a fully developed clitellum (indication of sexual maturity). Worms were then washed in distilled water to ensure the skin was free from soil and debris. The earthworms were then transferred to petri plate with Lumbricus Growth Medium (LGM) (1.25% agar, 0.31% gerber oatmeal, single grain (Nestle, Switzerland)) and incubated at 10°C for 48 hours to clear the digestive tract of any soil (Hutton et al., 2009 and Thesis) After worms were primed, the animals were selected for treatment. Prior to weighing,

worms were gently massaged along the length of the body to clear the gut of any digestive content. Six worms were selected with a consistent weight (± 1-1.5g), within a range of 5.0 to 6.0 g. Treatment plates contained concentrations of 0.5% w/v or 0.1% w/v of Lamiaceae herbs added to the standard LGM petri plate. Lamiaceae herbs were certified organic and free of contaminants. Eighteen earthworms were evenly divided into three groups and allowed to feed on the two different concentration plates for 2 and 6 days. They were then incubated at 18-20°C in the dark. After four days of treatment, a fresh LGM- Lamiaceae plate replaced the one consumed.

Removal of Seminal Vesicles

The earthworm's gut content was cleared by gently massaging the length of the body and each worm was weighed prior to anesthetization. They were rinsed in distilled water and placed in a glass petri plate and allowed to freeze for 30 minutes at -20 °C. A dissection was made along the dorsal side of the worm from the prostomium to the segments containing the seminal vesicles. The organ was gently removed and immediately transferred to a microcentrifuge tube containing 1 mL of Ca-free Lumbricus balanced salt solution (LBSS) and placed in an ice bath. The organ is then homogenized with a tissue tearor until a solution (without particles) is obtained. The homogenate is stored in an ice bath.

Evaluation of Sperm Quality Parameters

The sperm of *Lumbricus terrestris* were evaluated for abnormalities in morphology and the stages of developmental maturation as previously described by Zang et al. (Zang et al., 2000). 5 µL of the seminal vesicle homogenate was transferred onto a clean microscope slide and a cover slip placed on top avoiding air bubbles. 200

spermatozoa were counted under an inverted microscope at 40X objective. The number of mature (developed head and tail), immature (lack tail), and deformed sperm (apical loops, head bending) were noted. The percentage (%) of sperm deformity, maturity, and immaturity was calculated: % (deformed) = Average number of deformed sperm/ total number of sperm counted X 100. The same formula was used for calculating the percent of mature and immature sperm.

Preparation of Tissue Homogenate for Biochemical Analysis

The tissue homogenate is centrifuged at 1100 rpm for 2 minutes at 4°C. The supernatant was then transferred to a clean microcentrifuge tube and stored -80°C for future analyses. The remaining pellet is used for DNA fragmentation analysis.

DNA Fragmentation Analysis

This assay uses centrifugal sedimentation to separate fragmented double-stranded DNA from intact DNA (Martins-Cavagis, 2006). Upon lysis of spermatozoa, DNA is released and a centrifugation step will generate two fractions corresponding to intact and fragmented DNA (present in cytosol). Acid hydrolysis allows for deoxyribose sugars to bind with diphenylamine (DPA), and the percentage of fragmented DNA can be quantified spectrophotometrically. The pellet is first suspended in 0.5 mL of lysis buffer (5mM Tris-HCL Base, 1mM ethylenediaminetetraacetic acid (EDTA) Solution, 0.5% Triton-X 100; pH 9) and vortexed vigorously for 1-2 minutes. The solution is then centrifuged at 13000xg for 20 min at 4°C to separate intact and fragmented chromatin. The supernatant is transferred to another labeled microcentrifuge tube. The pellet is resuspended in lysis buffer and vortexed for 1-2 minutes. 20 µl of 6M perchloric acid is transferred to the tubes and vortexed for 1 minute to precipitate DNA. The mix is

incubated at room temperature for 5 minutes. The tubes are then centrifuged at 13000xg for 20 min at 4°C and the supernatant is discarded. The pellets were resuspended in 250 µl of 6M perchloric acid and vortexed vigorously for 1-2 minutes. The tubes were then incubated in a hot water bath at 70 °C for 20 minutes. 100 µl of DPA solution is then transferred to the tubes and incubated at 37 °C for 18 hours. 250 µl of the solution was transferred to a microplate and the absorbance was measured spectrophotometrically at 600nm. The percentage of fragmented DNA was calculated as follows: Fragmented DNA (%)= (Amount of the fragmented DNA in the supernatant) / (amount of the fragmented DNA in the supernatant + amount of DNA in the pellets) x 100.

Malondialdehyde Assay

This assay measures the lipid peroxidation by-product, Malondialdehyde (MDA), through its reaction with thiobarbutyric acid (Vattem et al., 2005). The resulting pink MDA/thiobarbutyric acid adduct can be measured spectrophotometrically. 150µl of the seminal vesicle extract supernatant was transferred to a test tube. The blank contained distilled water in place of the seminal vesicle extract supernatant. 100 µl of 20% (w/v) trichloroacetic acid was then transferred to the test tube. 200 µl of 10mM thiobarbutyric acid was also added to the test tubes. The test tubes were then covered and incubated in a hot water bath at 100 °C for 30 minutes. The content of the tubes was then transferred into a microcentrifuge tubes and centrifuge at 13000 rpm for 10 minutes at 4°C. 250 µl of the supernatant was transferred to a microplate and measured spectrophotometrically at an absorbance of 532nm. Calculation of the concentration of MDA is derived from its molar extinction coefficient, 156 µmol-1 cm-1. The concentration of MDA was expressed as mmol/mg of protein.

Protein Assay

This colorimetric protein assay is based on differential color changes of a dye in response to varied protein concentration derived from the method of Bradford (Bradford, 1976). 20 µl of seminal vesicle extract supernatant is transferred to a test tube. The blank contains distilled water in place of the seminal vesicle extract supernatant. 1ml of diluted dye reagent (Bio-Rad Protein Assay Kit, 1:4 DH_2O) is transferred to the test tube and vortexed. The mixture is incubated at room temperature for 5 minutes. The absorbance of the mix was measure spectrophotometrically at 595nm. The protein concentration was calculated based on a standard curve prepared using different dilutions of bovine serum albumin (BSA). The protein content of the sample was calculated using the slope obtained from the standard curve (0.0077). Protein content = A_{595}/ 0.0077/1000. The concentration of protein is expressed as mg/mL.

SOD-Riboflavin-NBT Assay

The superoxide dismutase activity was measured by its ability to prevent superoxide mediated oxidation nitroblue tetrazolium (NBT) to diformazan as a result of the photooxidation of riboflavin (Martinez et al., 2001). In this assay, 20 µL of seminal vesicle extract supernatant is transferred into a well of a 96 well microplate. 150 µL of riboflavin reaction mixture (2 mM riboflavin, 50 mM potassium phosphate (KH2PO4) buffer (pH 8.0), 0.1 mM EDTA, 200 µM diethylenetriamene pentaacetate (DTPA) and 57 µM NBT) was transferred to the well. 170 µL of riboflavin reaction mixture is transferred to a well to serve as the blank. The microplate is then incubated in a dark room and exposed to fluorescent lamps for 20 minutes. The absorbance is measured spectrophotometrically at 560nm. Calculation of the concentration of diformazan is

determined using its molar extinction coefficient, 26478 mol-1 cm-1. The concentration of diformazan was expressed as μmol/mg of protein.

Catalase Assay

Catalase activity is measured through a reaction initiated with the addition of 30% hydrogen peroxide in a sodium phosphate buffer (Sinha, 1972). The reaction is stopped with the addition of a potassium chromate-acetic acid reagent which initiates a color change with the remaining hydrogen peroxide. In this assay, 100 μL of seminal vesicle extract supernatant was transferred to a test tube. The blank contains Ca-Free LBSS in place of the seminal vesicle extract. Transfer 1.0 mL of peroxide reaction mixture (30% hydrogen peroxide, 0.1M sodium phosphate buffer) into the test tube. Incubate at room temperature for 10 minutes. Transfer 3.0 mL of potassium chromate-acetic acid reagent to the test tubes. Incubate the test tubes in a water bath at 100°C for 15 minutes. Let mixture cool at room temperature and transfer 250 μL into a 96 well microplate. The absorbance is measure spectrophotometrically at 570nm. Catalase activity was then calculated as follows: % decrease = $(A_{570}^{Blank} - A_{570}^{Treatment} / [A_{570}^{Blank}]) \times 100$. The concentration of catalase was expressed as mU/mg of protein.

Reduced Glutathione Determination

The seminal vesicle extract supernatant is placed in a precipitant solution to denature proteinaceous compounds. After a centrifugation step, the supernatant is assayed for nonprotein thiols, reduced glutathione (Mossman, 1983). A reduced glutathione-dithiobis nitrobenzoic acid (GSH-DTNB) complex can be measured spectrophotometrically at 412nm. 100 μL of seminal vesicle extract supernatant is

transferred to microcentrifuge tubes. 0.75 mL of precipitant solution is then added to the microcentrifuge tube. The mix is vortexed and incubated at room temperature for 5 minutes. The microcentrifuge tubes are then centrifuged at 3000xg for 15 minutes at 4°C. 500 µL of the supernatant is transferred to a test tube. 2.0 mL of 0.2M sodium phosphate (Na_2HPO_4) buffer (pH 8) is added to a small test tube. 250 µL of 0.5mM DTNB solution (Ellmans Reagent) is transferred to the test tube and vortexed. 250 µL of the mixture was immediately transferred to a microplate. The blank consisted of 500µL of 0.2M Na_2HPO_4 buffer (pH 8), 500µL DH_2O, 250µL of precipitant solution, 125µL of DTNB. The absorbance was reading spectrophotometrically at 412nm. The reduced glutathione concentration was calculated using the extinction coefficient *E*= 13.7 mol-1 cm-1(19) Molecular weight of GSH=307. The concentration of GSH was expressed as mmol/mg of protein.

Total Nitrate and Nitrite Determination

The assay described by Miranda et al. is used to reduce nitrate to nitrite by vanadium(III) chloride, followed by a spectrophotometric analysis of total nitrite using Griess reagent (Miranda et al., 2001). Colormetric methods based on the Griess reaction fundamentally detect NO2- that, under acidic conditions, reacts with sulfanilamide and NEDD to produce an azo compound, which strongly absorbs in the visible region with a peak around 545 nm. 50 µL of seminal vesicle extract supernatant was transferred to a well of a microplate. The blank contains Ca-free LBSS in place of seminal vesicle extract supernatant. 50 µL of 0.8% (w/v) vanadium chloride was added to the well. 50 µL of Greiss Reagent (if premade) was transferred to the well of the microplate. If Griess reagent isn't available, use 25 µL sulfanilamide (2%) and 25µL N-(1-Naphthyl)

ethylendiamine dihydrochloride (0.1%). The mixture was incubated in a microplate reader at 37^0C for 30 minutes and the absorbance measured at 540 nm. Calculation of NOx in serum samples was determined from linear standard curve established by 0-150 umol/l sodium nitrate. Concentration (umol/L) = (A_{540}- 0.0344)/0.0057. The concentration of NOx was expressed as μm/L/mg of protein.

Statistical Analysis

Statistical analysis of data was performed using a two tailed Student's *t* test. The data are presented as means ± standard error mean (SEM). p values ≤ 0.05 were considered statistically significant. Additionally, to measure the overall effectiveness of treatment over the duration of 6 days the area under the curve (AUC) was calculated.

Results

Sperm Deformations

Free radicals are known to cause deformations in spermatozoa. These deformed cells are also able to contribute to the generation of ROS within their environment. Evaluation of sperm deformations after a 6 day treatment with Lamiaceae herbs revealed significant changes in the ratio of deformed to normal cells. *Lumbricus terrestris* which only consumed lumbricus growth medium (LGM) for 6 days were established as controls in this study. After two days of exposure, 19.3% of spermatozoa exhibited a deformation. On day 6, 20.1% of all spermatozoa evaluated had some type of deformation in control worms (Table 1).

Among all Lamiaceae herbs tested, basil was the most effective herb in terms of its ability to decrease sperm deformations. At 0.1% (w/v) of basil, deformations were 6% and 13% on day 2 and day 6 respectively, which was significantly (p=0.0000; p=0.0001)

lower than controls (Table 2). At the concentration of 0.5% (w/v), sperm deformities were calculated to be 15.3% and 9.4% on day 2 and day 6 respectively, which translates to significantly (p=0.0003; p=0.0001) lower deformations when compared to controls (Table 2). The overall effectiveness of treatment was determined by calculating the area under the curve (AUC). It was observed that treatment with basil at 0.1% (w/v) was significantly (p=0.0095) more effective at reducing morphological deformations in spermatozoa than the treatment at 0.5% (w/v) over the span of 6 days (Figure 1).

Thyme was the next best herb at preventing deformations in sperm in Lumbricus terrestris. At the concentration of 0.1% (w/v), deformities were calculated to be 9.8% and 13.0% on day 2 and day 6 respectively, which is significantly (p=0.0004; p=0.0013) lower than controls (Table 2). At 0.5% (w/v) of thyme, sperm deformations were 6.4% and 8.5% on day 2 and day 6 respectively, which were significantly (p=0.0007; p=0.0003) lower when compared to controls (Table 2). Thyme at 0.5% (w/v) was significantly (p=0.0056) more effective at preventing sperm deformations than treatment at 0.1% (w/v) over the duration of 6 days (Figure 1).

Treatment with rosemary was not as effective as the previous herbs in terms of reducing sperm deformations. With the concentration at 0.1% (w/v), deformations on day 2 were 11.1% and 13.0% on day 6, which was significantly (p=0.0005; p=0.0001) lowered with respect to control values (Table 2). Rosemary at 0.5% (w/v), resulted in sperm deformations at 9.6% and 7.6% on day 2 and day 6 respectively, which were also less than control values (p=0.0068; p=0.0002) (Table 2). Upon observation of total effectiveness of treatment, it was revealed that 0.5% (w/v) resulted in a greater reduction (p=0.0075) in sperm deformations than treatment at 0.1% (w/v) (Figure 1).

Oregano and sage were the least effective Lamiaceae herbs at reducing the number of deformed sperm within seminal vesicles. Oregano at 0.1% (w/v), resulted in deformations at 12.2% and 16.6% on day 2 and day 6 respectively, with a decrease in deformations on day 2 in comparison to the control (p=0.0016) (Table 2). With oregano at 0.5% (w/v), the percentages of deformities were 18.3% and 15.2% on day 2 and day 6 respectively, which means a significant (p=0.0262) decrease in deformations occurred on day 6 (Table 2). It was shown that oregano at 0.1% (w/v) was significantly (p=0.0167) more effective at reducing sperm deformations than the treatment at 0.5% (w/v) over the course of 6 days (Figure 1). Sage was the least effective herb in reducing morphological deformations in spermatozoa. At 0.1% (w/v), deformations were calculated to be 22.3% and 13.0% on day 2 and day 6 respectively, with decreases in deformations on day 6 when compared to controls (p=0.0059) (Table 2). With sage at 0.5% (w/v), the percentages of deformations were 17.6% on day 2 and 11.5% on day 6, which were both significantly (p=0.0435; p=0.0003) decreased when compared to control values (Table 2). Sage at the higher concentration of 0.5% (w/v) was significantly (p=0.0130) more effective in lowering sperm deformations from occurring than treatment at 0.1% (w/v) over the duration of treatment (Figure 1).

Table 2: Effect of Different Lamiaceae Herbs on Sperm Deformations.
* indicates significantly lower than control ($p< 0.05$); n=7

Concentration %(w/v)		Sperm Deformations (%)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	6.0	0.0001*	13.0	0.0001*
	0.5	15.3	0.0003*	9.4	0.0001*
Oregano	0.1	12.2	0.0016*	16.6	0.0779
	0.5	18.3	0.3653	15.2	0.0262*
Rosemary	0.1	11.1	0.0005*	13.0	0.0005*
	0.5	9.6	0.0068*	7.6	0.0002*
Sage	0.1	22.3	0.0524	13.0	0.0059*
	0.5	17.6	0.0435*	11.5	0.0003*
Thyme	0.1	9.8	0.0004*	13.0	0.0013*
	0.5	6.4	0.0007*	8.5	0.0003*
Control	LGM	19.3	-	20.1	-

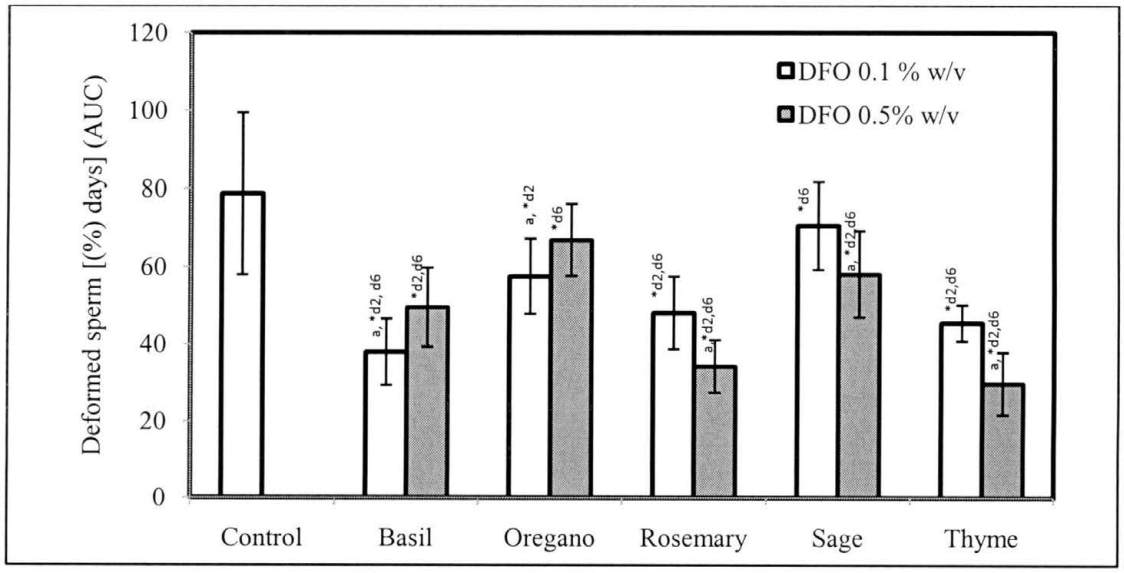

Figure 1: Overall Effectiveness of Treatment with Lamiaceae Herbs on Sperm Deformations (DFO) Over the Duration of 6 Days.
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Malondialdehye Assay

The extent of lipid peroxidation in seminal vesicles was measured by assessing the amount of malondialdehyde formed. In control worms (no treatment) the levels of MDA after 2 days on LGM was 1.3 mmol/mg of protein. On the 6th day, the level of MDA increased to 2.2 mmol/mg of protein. Over the course of 6 days the amount of MDA in the controls was 6.9 mmol/mg of protein.

Among all the Lamiaceae herbs tested, basil was most effective in lowering the extent of lipid oxidation over the duration of the treatment. At 0.1% (w/v) of basil, the MDA values were 0.2 mmol/mg of protein and |0.0| mmol/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0055; p= 0.0000) lower than the control (Table 3). At the basil concentration of 0.5% (w/v), the levels of MDA were 2.9 mmol/mg of protein on day 2 and 0.1 mmol/mg of protein on day 6. Lipid oxidation significantly increased (p=0.0000) on day 2 and decreased (p=0.0000) on day 6 in comparison to the control (Table 3). It was observed that treatment with basil at a concentration of 0.1% (w/v) (AUC= 0.0172 mmol/mg of protein) was significantly (p=0.0013) more effective in reducing the MDA levels than the concentration at 0.5% (w/v) (AUC= 6.0053 mmol/mg) over the duration of treatment (Figure 2).

Rosemary was the next most effective herb in terms of its ability to lower lipid oxidation. At 0.1% (w/v), the MDA levels were 0.2 mmol/mg of protein and 0.0 mmol/mg of protein on day 2 and day 6 respectively, which translates to levels significantly (p=0.0011; p= 0.0000) lower than the controls (Table 3). Treatment at 0.5% (w/v), resulted in levels of MDA at 0.5 mmol/mg of protein and 0.3 mmol/mg of protein on day 2 and day 6 respectively, which were also attenuated compared to controls

(p=0.0011; p= 0.0000) (Table 3) . It was shown that rosemary at 0.1% (w/v) (AUC= 0.3512 mmol/mg of protein) was significantly (p=0.0019) more effective in reducing MDA values than the treatment at 0.5% (w/v) (AUC=1.5752 mmol/mg) (Figure 2).

Oregano was the third best herb in its ability to reduce MDA formation. With the treatment at 0.1% (w/v), MDA values were 0.3 mmol/mg of protein and 0.6 mmol/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.00324; p= 0.00004) blunted compared to controls (Table 3). At the increased concentration of 0.5% (w/v), the lipid oxidation byproducts were 0.6 mmol/mg of protein on day 2 and 0.2 mmol/mg of protein on day 6. Again, significant (p= 0.01492; p=0.00002) reductions in MDA were observed (Table 3). Over the extent of the six day treatment it was revealed that the concentration at 0.1% (w/v) (AUC=1.6509 mmol/mg of protein) was significantly (p=0.03383) more effective at attenuating MDA levels than 0.5% (w/v) (AUC= 1.7776 mmol/mg) (Figure 2).

The least effective of the Lamiaceae herbs at reducing lipid oxidation were thyme and sage. Thyme at 0.1% (w/v), resulted in levels of MDA of 0.4 mmol/mg of protein and 0.5 mmol/mg of protein on day 2 and day 6 respectively, with significant (p=0.0010; p= 0.0000) decreases in comparison to controls (Table 3). With the concentration of thyme at 0.5% (w/v), the levels of MDA were 0.6 mmol/mg of protein on day 2 and 0.4 mmol/mg of protein on day 6, which was also significantly (p=0.0022; p= 0.0000) lower than controls (Table 3). It was observed that thyme at 0.1% (w/v) (AUC=1.6562 mmol/mg of protein) was significantly (p=0.0125) more effective than 0.5% (w/v) (AUC= 2.0229 mmol/mg of protein) in countering lipid oxidation in seminal vesicles (Figure 2). Among the Lamiaceae herbs, sage wasn't quite effective as the previous four

herbs in its ability to lower MDA formation. The sage concentration at 0.1% (w/v) resulted in levels of MDA of 0.3 mmol/mg of protein and 0.8 mmol/mg of protein on day 2 and day 6 respectively, which were observed to be significantly (p=0.0002; p= 0.0000) lowered in comparison to controls (Table 3). Treatment at 0.5% (w/v), revealed lipid oxidation byproduct values of 0.5 mmol/mg of protein on day 2 and 0.1 mmol/mg of protein on day 6 (Table 3). Again, significant decreases in MDA formation were noted (p=0.006; p= 0.0000). Significantly higher (p=0.0036) levels of MDA at 0.1% (w/v) (AUC= 2.2743 mmol/mg of protein) revealed that treatment at 0.5% (w/v) (AUC=1.0999 mmol/mg) was more effective at reducing lipid oxidation over the 6 days of treatment (Figure 2).

Table 3: Effect of Different Lamiaceae Herbs on MDA (mmol/mg of protein) Formation in Seminal Vesicles as Measured by TBARS.
* indicates significantly lower than control; #significantly higher than control ($p< 0.05$); n=7

Concentration % (w/v)		MDA (mmol/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	0.2	0.0055*	-0.2	0.0000*
	0.5	2.9	0.0000#	0.1	0.0001*
Oregano	0.1	0.3	0.0032*	0.6	0.0000*
	0.5	0.6	0.0149*	0.2	0.0000*
Rosemary	0.1	0.2	0.0011*	0.0	0.0000*
	0.5	0.5	0.0001*	0.3	0.0000*
Sage	0.1	0.3	0.0002*	0.8	0.0001*
	0.5	0.5	0.0061*	0.1	0.0000*
Thyme	0.1	0.4	0.0011*	0.5	0.0001*
	0.5	0.6	0.0022*	0.4	0.0001*
Control	LGM	1.3	-	2.2	-

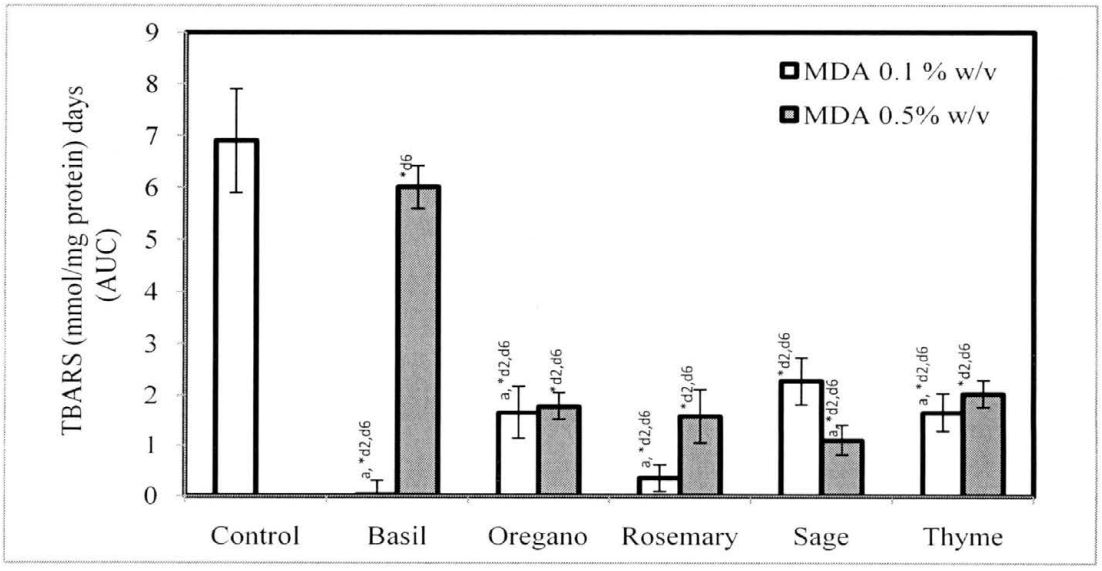

Figure 2: Overall Effectiveness of Different Lamiaceae Herbs on Levels of MDA in Seminal Vesicles Over 6 Days Measured as TBARS
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means $\pm$ SEM

DNA Fragmentation

In order to assess the extent of free radical induced damage to spermatozoa, the percentage of DNA fragmentation was measured. In control worms that fed on LGM , it was shown that on the second day of treatment the amount of DNA fragmentation was 43.2 %. On day 6, the extent of fragmentation decreased to 41.0%.

Treatment with rosemary proved to be most effective in reducing DNA fragmentation among all the Lamiaceae herbs tested. With rosemary at 0.1% (w/v), the amount of fragmented DNA was observed to be at 39.6 % and 38.1% on day 2 and day 6 respectively, which was significantly (p=0.0124; p=0.0009) less than control values (Table 4). When the concentration was increased to 0.5% (w/v), the percentages of DNA fragmentation were 31.1% on day 2 and 48.4% on day 6. A significant (p=0.0003) decrease in fragmentation was prevalent on day 2, but significant (p=0.0002) increases in fragmentation occurred on day 6 when compared to controls (Table 4). It was observed that the amount of DNA fragmentation at 0.1% (w/v) was not significantly different than the concentration at 0.5% (w/v) over the course of treatment as determined by AUC values (Figure 3).

Oregano was the second best herb at preventing fragmentation in the DNA. Tests conducted with 0.1% (w/v) revealed fragmentations of 41.7% and 36.7% on day 2 and day 6 respectively, with significant reductions occurring on the second day when compared to controls (p=0.0092) (Table 4). At 0.5% (w/v), the extent of fragmented DNA was 36.4% and 35.1% on day 2 and day 6 respectively, which were both significantly (p=0.0017; p=0.0033) lower than controls (Table 4). Overall, oregano at

0.5% (w/v) was significantly (p=0.0274) more effective at preventing DNA strand breaks than at 0.1% (w/v) as determined by the AUC (Figure 3).

Sage was able to prevent DNA fragmentation to a lesser extent than the previous two herbs. Sage at the concentration of 0.1% (w/v), resulted in fragmentation of 45.2% and 45.0% in the DNA on day 2 and day 6 respectively, which was significantly (p=0.0263; p=0.0066) higher than controls (Table 4). Sage at 0.5% (w/v), resulted in fragmentation of 40.4% and 48.9% on day 2 and day 6 respectively, which was shown to significantly (p=0.0121) decrease the amount of DNA fragmentation on day 2 when compared to controls. However, on day 6 the fragmentation was significantly (p=0.0000) higher than controls (Table 4). Overall, it has been observed that the amount of DNA fragmentation at 0.1% (w/v) was not significantly different than the concentration at 0.5% (w/v) as determined by the AUC (Figure 3).

Among all Lamiaceae herbs, basil and thyme were the least effective in terms of their ability to lower DNA fragmentation. At 0.1% (w/v) of basil, fragmentation in DNA was 48.6% and 41.7% on day 2 and day 6 respectively, with significant (p=0.0046) increases in the amount of DNA fragmentation on day 2 when compared to the controls (Table 4). With the increased basil concentration at 0.5% (w/v), the extent of DNA damage was 43.9% on day 2 and 40.9% on day 6 (Table 4). Over the duration of 6 days AUC values indicated that the concentration at 0.5% (w/v) had significantly (p=0.0402) less DNA fragmentation than at 0.1% (w/v) (Figure 3). Thyme was the least effective herb at lowering the levels of DNA fragmentation. The concentration of thyme at 0.1% (w/v), resulted in DNA fragmentations of 44.7% and 47.9% on day 2 and day 6 respectively, which were significantly (p=0.0239; p=0.0000) higher than controls (Table

4). The results with thyme at 0.5% (w/v) were similar, and had DNA fragmentation levels of 46.6% on day 2 and 47.1% on day 6 which were both significantly (p=0.0098; p=0.0005) higher in comparison to control values (Table 4). It was concluded from AUC values that treatment at 0.1% (w/v) was not significantly different than treatment at 0.5% (w/v) (Figure 3).

Table 4: Effect of Different Lamiaceae Herb Treatments on DNA Fragmentation (%)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		DNA Fragementation (%)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	48.6	0.0046#	41.7	0.3772
	0.5	43.9	0.2593	40.9	0.4797
Oregano	0.1	41.7	0.0092*	36.7	0.0658
	0.5	36.4	0.0017*	35.1	0.0033*
Rosemary	0.1	39.6	0.0124*	38.1	0.0009*
	0.5	31.1	0.0003*	48.4	0.0002#
Sage	0.1	45.2	0.0263#	45.0	0.0066#
	0.5	40.4	0.0121*	48.9	0.0000#
Thyme	0.1	44.7	0.0239#	47.9	0.0000#
	0.5	46.6	0.0098#	47.1	0.0005#
Control		43.2	-	41.0	-

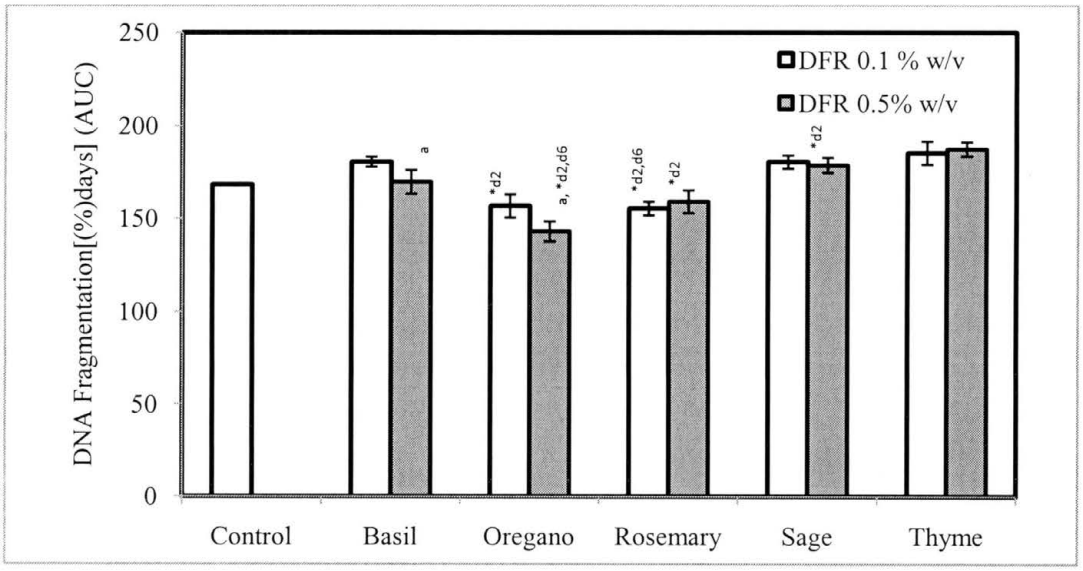

Figure 3: Overall Effectiveness of Lamiaceae Treatment on Levels of DNA Fragmentation (DFR) Over 6 Days
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Superoxide Dismutase

The superoxide dismutase (SOD) activity was measured by the ability of the seminal vesicle extract in preventing superoxide mediated oxidation of NBT to diformazan as a result of the photooxidation of riboflavin. In control worms on day 2, the level of diformazan formed was 126.1 μmol/mg of protein. Tests on day 6 revealed that the levels of diformazan had decreased to 81.4 μmol/mg of protein.

Rosemary was the most effective herb at reducing diformazan formation. Treatment with rosemary at a concentration of 0.1% (w/v), resulted in diformazan levels of 65.3 μmol/mg of protein and 56.4 μmol/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.0000;p=0.0000) lower in comparison to control values (Table 5). With the rosemary concentration at 0.5% (w/v), diformazan levels were 104.5 μmol/mg of protein on day 2 and 76.0 μmol/mg of protein on day 6, which was also significantly lower than controls on day 2 (p=0.0008) and on day 6 (p=0.0005) (Table 5). Upon determination of the AUC it was observed that the concentration of rosemary at 0.5% (w/v) was significantly (p=0.0084) more effective at preventing diformazan formation than the concentration at 0.1% (w/v) (Figure 4).

Sage was the next best herb at increasing SOD activity as indicated by lower levels of diformazan. Treatment with sage at 0.1% (w/v) resulted in levels of diformazan at 78.0 μmol/mg of protein and 93.0 μmol/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.0002) lower on day 2 and significantly (p=0.0001) higher on day 6 in comparison to controls (Table 5). At 0.5% (w/v) of sage, diformazan levels were 121.9 μmol/mg of protein on day 2 and 116.7 μmol/mg of protein on day 6, which was significantly (p=0.0110) lower on day 2 and significantly (p=0.0001) higher on day 6

in comparison to controls (Table 5). It was observed that the concentration of sage at 0.5% (w/v) was significantly (p=0.0070) more effective at reducing levels of diformazan than the concentration of 0.1% (w/v) over the duration of treatment as determined by AUC values (Figure 4).

Basil was not as effective as rosemary and sage in terms of increasing SOD activity. Treatment with basil at 0.1% (w/v), resulted in diformazan levels of 90.6 µmol/mg of protein and 85.6 µmol/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.0005) lower on day 2 and significantly (p=0.0313) higher on day 6 in comparison to controls (Table 5). At 0.5% (w/v) of basil, diformazan levels were 99.0 µmol/mg of protein on day 2 and 91.5 µmol/mg of protein on day 6. On day 2 levels of diformazan were significantly (p=0.0013) lower than controls, but on day 6 levels were significantly (p=0.0008) higher (Table 5). Based on AUC values it was noted that treatment with basil at 0.5% (w/v) was significantly (p=0.0042) more effective at lowering diformazan levels than the concentration at 0.1% (w/v) over 6 days (Figure 4).

Thyme and oregano were least effective out of all Lamiaceae herbs in their ability to prevent diformazan formation. Thyme at 0.1% (w/v), resulted in diformazan levels of 90.7 µmol/mg of protein on day 2 and 105.9 µmol/mg of protein on day 6. There was a significant (p=0.0003) decrease in diformazan formation on day 2 and a significant (p=0.0155) increase on day 6 in comparison to the control values (Table 5). At 0.5% (w/v) of thyme, diformazan levels were 99.1 µmol/mg of protein and 91.3 µmol/mg of protein on day 2 and day 6 respectively, with significantly (p=0.0013) lower levels on day 2 and significantly (p=0.0042) higher levels on day 6 when compared to controls (Table 5). Over the duration of 6 days, thyme at 0.5% (w/v) was significantly (p=0.0036)

more effective than the concentration of 0.1% ((w/v)) in preventing diformazan formation as indicated by AUC values (Figure 4). Oregano was the least effective in its ability prevent diformazan formation. At 0.1% (w/v), diformazan levels were 118.7 μmol/mg of protein and 129.7 μmol/mg of protein on day 2 and day 6 respectively, with significant (p=0.0000) increases in diformazan levels in comparison to control values on day 6 (Table 5). With the concentration of oregano at 0.5% (w/v), the levels of diformazan were 87.1 μmol/mg of protein on day 2 and 117.8 μmol/mg of protein on day 2. At this concentration on day 2, oregano was able to significantly (p=0.0000) lower diformazan formation, but on day 6 diformazan concentrations increased significantly (p=0.0000) in comparison to the control (Table 5). It was observed from the AUC values that the concentration of oregano at 0.5% (w/v) was significantly (p=0.0003) more effective at decreasing diformazan levels than the concentration of 0.1% (w/v) (Figure 4).

Table 5: Effect of Lamiaceae Herbs on Superoxide Dismutase Activity as Indicated by Levels of Diformazan (μmol/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Diformazan (μmol/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	90.6	0.0006*	85.6	0.0313#
	0.5	99.0	0.0014*	91.5	0.0008#
Oregano	0.1	118.7	0.0699	129.7	0.0000#
	0.5	87.1	0.0000*	117.8	0.0000#
Rosemary	0.1	65.3	0.0000*	56.4	0.0000*
	0.5	104.5	0.0008*	76.0	0.0005*
Sage	0.1	78.0	0.0002*	93.0	0.0001#
	0.5	121.9	0.0110*	116.7	0.0001#
Thyme	0.1	90.7	0.0003*	105.9	0.0155#
	0.5	99.1	0.0013*	91.3	0.0042#
Control	LGM	126.1	-	81.4	-

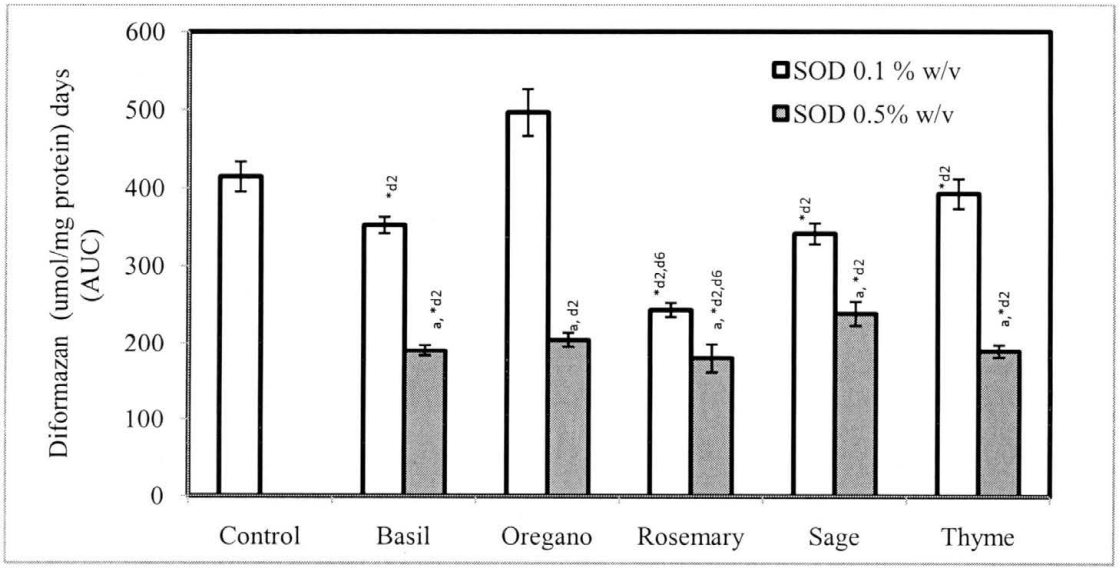

Figure 4: Overall Effectiveness of Lamiaceae Treatment on Superoxide Dismutase Activity as Indicated by Levels of Diformazan Over 6 Days
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Catalase

Removal of hydrogen peroxide, a powerful reactive oxygen species and product of SOD mediated reactions, requires the enzymatic action of catalase. The observed reductions in MDA and DNA fragmentation prompted the investigation of the effects of Lamiaceae herbs on catalase activity. In control worms which fed only on LGM, the catalase activity was 117.9 mU/ mg of protein after two days. The catalase activity after six days of treatment was 130.4 mU/mg of protein.

The most effective Lamiaceae herb at increasing catalase activity over the duration of treatment was basil. With the basil concentration at 0.1% (w/v), catalase activity was 157.3 mU/mg of protein and 154.7 mU/mg of protein on day 2 and day 6 respectively, which indicated significant (p=0.0233; p=0.0410) increases when compared to controls (Table 6). At 0.5% (w/v), the catalase activity was 145.8 mU/mg of protein on day 2 and 144.2 mU/mg of protein on day 6. On day 6 there was a significant (p=0.0050) decrease in catalase activity when compared to controls (Table 6). It was observed that the concentration of basil at 0.1% (w/v) was significantly (p=0.0342) more effective in increasing catalase activity than 0.5% (w/v) over the duration of treatment as noted from AUC values (Figure 5).

Oregano was the second best herb at increasing catalase activity. Treatment at 0.1% (w/v) of oregano, resulted in catalase activity of 151.1 mU/mg of protein and 133.3 mU/mg of protein on day 2 and day 6 with respectively, with significant (p=0.0000; p=0.0041) increases in comparison to controls (Table 6). At the concentration of 0.5% (w/v), the catalase activity at day 2 was 95.6 mU/mg of protein and on day 6 112.6

mU/mg of protein. The only significant (p=0.0089) increase in catalase activity occurred on day 2 (Table 6). Upon calculation of the AUC, it was observed that over the duration of treatment, catalase activity was significantly (p=0.0080) higher at 0.1% (w/v) than at 0.5% (w/v) (Figure 5).

Sage was not as effective as oregano and basil in its ability to increase catalase activity. The sage concentration at 0.1% (w/v) resulted in catalase activity of 171.3 mU/mg of protein and 109.3 mU/mg of protein on day 2 and day 6 respectively. Opposite effects with this treatment occurred on both days with significant (p=0.0006) increases on day 2 and decreases (p=0.0089) on day 6 upon comparison to controls (Table 6). At 0.5% (w/v) of sage, the catalase activity was 86.8 mU/mg of protein and 114.9 mU/mg of protein on day 2 and 6 respectively, which was significantly (p=0.0000; p= 0.0025) decreased when compared to controls (Table 6). It was observed that sage at 0.1% (w/v) was significantly (p=0.0077) more effective than 0.5% (w/v) in upregulating catalase activity over the duration of the treatment (Figure 5).

Thyme and rosemary were least effective at generating increases in catalase activity. Thyme at 0.1% (w/v) resulted in catalase activity of 117.4 mU/mg of protein and 162.2 mU/mg of protein on day 2 and day 6 respectively (Table 6). At the thyme concentration of 0.5% (w/v), the catalase activity at day 2 was 122.1 mU/mg of protein and on day 6 it was 143.5 mU/mg of protein. The results indicate that catalase activity significantly increased on day 2 (p=0.0363) in comparison to the control (Table 6). Among all Lamiaceae herbs tested rosemary was least effective at increasing catalase activity. At 0.1% (w/v) of rosemary, the catalase activity was 118.7 mU/mg of protein and 87.1 mU/mg of protein on day 2 and day 6 respectively. Increasing the rosemary

concentration to 0.5% (w/v) resulted in catalase activity of 129.7 mU/mg of protein on day 2 and 117.8 mU/mg of protein on day 6. Treatment at this concentration resulted in significant (p=0.0005; p=0.0036) decreases in catalase activity when compared to controls (Table 6). From the AUC values, it was noted that the concentration at 0.1% (w/v) was significantly (p=0.0465) more effective at increasing catalase activity than 0.5% (w/v) (Figure 5).

Table 6: Effect of Different Lamiaceae Herbs on Catalase Activity (mU/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Catalase activity (mU/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	157.3	0.0233*	154.7	0.0410*
	0.5	145.8	0.0922	144.2	0.0051#
Oregano	0.1	151.1	0.0000*	95.6	0.0041*
	0.5	133.3	0.0089*	112.3	0.2575
Rosemary	0.1	115.3	0.4215	85.6	0.2558
	0.5	152.9	0.0005#	106.1	0.0036#
Sage	0.1	171.3	0.0006*	109.3	0.0089#
	0.5	86.8	0.0001#	114.9	0.0025#
Thyme	0.1	117.4	0.0839	122.1	0.3527
	0.5	162.2	0.0363*	143.5	0.0846
Control	LGM	117.9	-	130.4	-

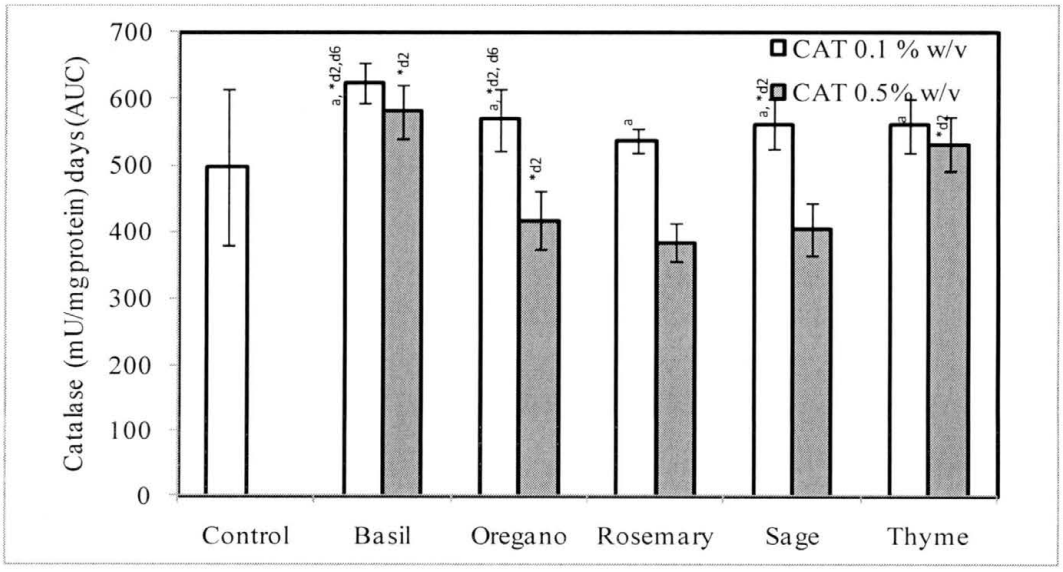

Figure 5: Overall Effectiveness of Different Lamiaceae Herbs on Catalase Activity Over 6 Days
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Glutathione

Glutathione plays an important role in many biological processes and is a critical cellular antioxidant involved in maintaining cytosolic redox homeostasis. In control worms, on day 2 the level of glutathione was 68.1 mmol/mg of protein. On day 6 the amount of glutathione increased to 71.1 mmol/mg of protein.

Among all Lamiaceae herbs, oregano was most effective at increasing levels of glutathione when compared to controls. With oregano at 0.1% (w/v), observed levels of glutathione were 96.8 mmol/mg of protein and 73.3 mmol/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0092) higher than controls on day 2 (Table 7). Treatment at the increased concentration of 0.5% (w/v), had glutathione levels of 72.5 mmol/mg of protein on day 2 and 78.1 mmol/mg of protein on day 6. At this concentration, levels of glutathione were significantly (p=0.0066; p=0.0033) higher than controls on day 2 and day 6 (Table 7). From AUC values it was noted that treatment with oregano at 0.1% (w/v) was significantly (p=0.0205) more effective at increasing gluthatione levels than at 0.5% (w/v) over the duration of 6 days (Figure 6).

Sage proved to be the next best herb in terms of its ability to increase glutathione levels. At 0.1% (w/v) of sage, glutathione levels were 75.3 mmol/ mg of protein on day 2 and 65.7 mmol/ mg of protein on day 6. Levels of glutathione were significantly (p= 0.0003) higher than controls on day 2 (Table 7). Increasing the sage concentration to 0.5% (w/v), resulted in glutathione levels of 62.5 mmol/mg of protein and 64.9 mmol/mg of protein on day 2 and day 6 respectively, which was significantly (p= 0.0001) lower levels than controls on day 2 (Table 7). It was observed that the glutathione levels at

0.1% (w/v) were significantly (p=0.0246) higher than at 0.5% (w/v) over the 6 days of treatment based on AUC values (Figure 6).

Thyme was not as effective as oregano and sage in its ability to increase glutathione levels. Treatment with thyme at 0.1% (w/v), resulted in levels of glutathione at 65.7 mmol/ mg of protein and 65.6 mmol/ mg of protein on day 2 and day 6 repectively, which was significantly (p= 0.0146) lower than controls on day 2 (Table 7). Increasing the treatment concentration to 0.5% (w/v) resulted in glutathione levels at 64.2 mmol/ mg on day 2 and 69.2 mmol/ mg on day 6. Again, significantly (p= 0.0006) lower levels of glutathione were seen on day 2 in comparison to controls (Table 7). The overall effectiveness of treatment at 0.1% (w/v) was not significantly different than the concentration at 0.5% (w/v) (Figure 6).

Basil and rosemary were the least effective Lamiaceae herbs in their ability to increase levels of glutathione. Basil at 0.1% (w/v), had levels of glutathione at 61.9 mmol/ mg of protein and 57.8 mmol/ mg of protein on day 2 and day 6 respectively, which were significantly (p= 0.0003; p=0.0153) lower than control values (Table 7). At 0.5% (w/v) of basil, glutathione levels were 66.0 mmol/mg of protein on day 2 and 60.9 mmol/mg of protein on day 6. On the 6^{th} day of treatment glutathione levels were significantly (p=0.0460) lower than controls (Table 7). It was observed that treatment with basil at 0.1% (w/v) had significantly (p=0.0422) lower levels of glutathione than at 0.5% (w/v) over the span of treatment (Figure 6). Among all Lamiaceae herbs, rosemary was least effective at increasing levels of glutathione. At 0.1% (w/v), glutathione levels were 54.5 mmol/mg of protein and 58.4 mmol/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.0001; p=0.0190) lower than control values

(Table 7). With rosemary at 0.5% (w/v), it was observed that levels of glutathione were 57.2 mmol/mg of protein on day 2 and 58.2 mmol/mg of protein on day 6. Again, these values were significantly (p=0.0000; p= 0.0217) lower in comparison with control levels of glutathione (Table 7). It was shown that glutathione levels at 0.1% (w/v) were not significantly different than the concentration at 0.5% (w/v) over the course of treatment as indicated by AUC values (Figure 6).

Table 7: Effect of Lamiaceae Herbs on Levels of Glutathione in the Tissues of Seminal Vesicles

* indicates significantly lower than control; #significantly higher than control($p<0.05$); n=7

Concentration % (w/v)		Glutathione (mmol/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	61.9	0.0003#	57.8	0.0153#
	0.5	66.0	0.0671	60.9	0.0460#
Oregano	0.1	96.8	0.0092*	73.3	0.0658
	0.5	72.5	0.0017*	78.1	0.0033*
Rosemary	0.1	54.5	0.0001#	57.2	0.0190#
	0.5	58.4	0.0000#	58.2	0.0217#
Sage	0.1	75.3	0.0003*	65.7	0.0758
	0.5	62.5	0.0001#	64.9	0.1318
Thyme	0.1	65.7	0.0146#	65.6	0.1854
	0.5	64.2	0.0006#	69.2	0.3592
Control	LGM	68.1	-	71.1	-

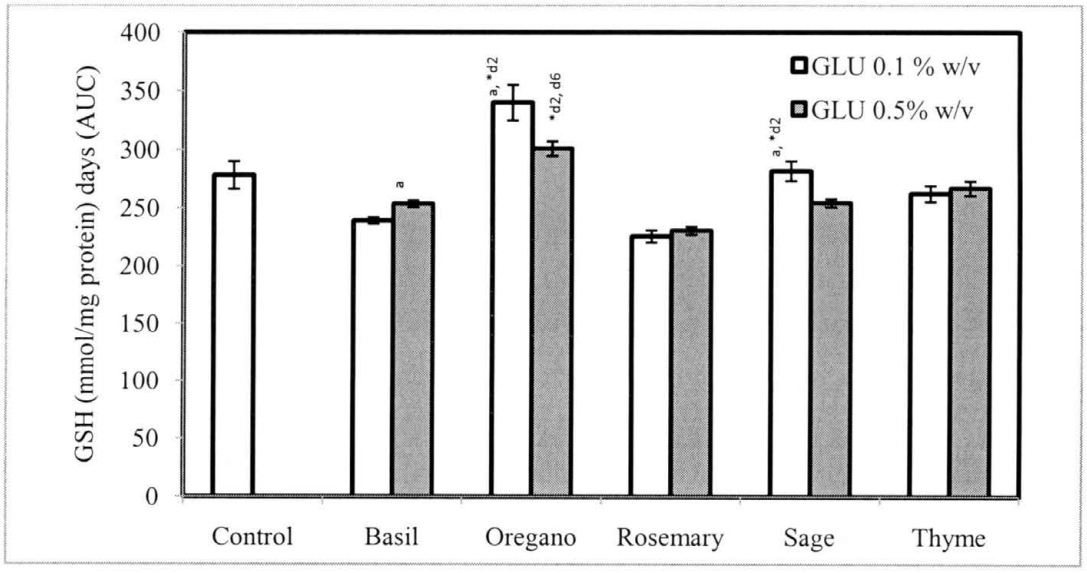

Figure 6: Overall Effectiveness of Different Lamiaceae Herbs on Glutathione Levels (GSH, GLU) over 6 days

a- indicates the treatment concentration which was significantly different ($p<0.05$)

*- indicates significant difference from the control ($p<0.05$)

d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)

The data is represented as means $\pm$ SEM

Nitric Oxide

Nitric oxide plays a significant role in cell signaling and vasodilation in reproductive physiology. The effect of Lamiaceae herbs on nitric oxide production in seminal vesicles was analyzed indirectly by determining total nitrate/nitrite levels using a standard Griess test. Control worms had total levels of nitric oxide at 357.0 µmol/L/mg of protein and 196.9 µmol/L/mg of protein on day 2 and day 6 respectively.

Rosemary was the best herb in its ability raise nitric oxide levels in seminal vesicles over the duration of treatment. Upon treatment with rosemary at 0.1% (w/v) resulted in total nitric oxide values of 358.6 µmol/L/mg of protein and 333.8 µmol/L/mg of protein were observed on day 2 and day 6 respectively, with significantly (p=0.0002) higher levels on day 6 in comparison to controls (Table 8). At 0.5% (w/v), the total nitric oxide values were 286.4 µmol/L/mg of protein on day 2 and 203.9 µmol/L/mg of protein on day 6 (Table 8). From AUC calculations rosemary at 0.1% (w/v) was significantly (p=0.0073) more efficient at increasing nitric oxide than 0.5% (w/v) over 6 days of treatment (Figure 7).

Basil was the next most effective herb at stimulating the production of nitric oxide over the duration of 6 days. With basil at 0.1% (w/v), the total nitric oxide values were 310.1 µmol/L/mg of protein and 373.0 µmol/L/mg of protein on day 2 and day 6 respectively, with significantly (p=0.0023) lower values on day 2 and higher (p=0.0006) values on day 6 when compared to controls (Table 8). At the basil concentration of 0.5% (w/v), the nitric oxide values were 294.0 µmol/L/mg of protein on day 2 and 299.0 µmol/L/mg of protein on day 6. Results were similar to the previous concentration of 0.1% (w/v) with a significant (p=0.0006) decrease on day 2 and increase (p=0.0000) in

nitric oxide on day 6 (Table 8). It was shown that at 0.1% (w/v), total nitric oxide was significantly (p=0.0177) higher than values at 0.5% (w/v) over the duration of treatment (Figure 7).

Treatment with oregano was moderate in its effect on the production of nitric oxide over the course of 6 days. At 0.1% (w/v) of oregano, total nitric oxide levels were 264.3 µmol/L/mg of protein and 408.4 µmol/L/mg of protein on day 2 and day 6 respectively, which were significantly (p=0.0046) decreased on day 2 and increased (p=0.0000) on day 6 in comparison to control levels (Table 8). At the oregano concentration of 0.5% (w/v), the nitric oxide production was 269.9 µmol/L/mg of protein and 311.0 µmol/L/mg of protein on day 2 and day 6 respectively. Again, the results indicated significantly (p=0.0068) lower values on day 2 and higher (p=0.0000) values on day 6 when compared to controls (Table 8). Oregano treatment at 0.1% (w/v) was capable of significantly (p=0.0171) stimulating more nitric oxide production than the concentration at 0.5% (w/v) over the duration of treatment as determined by the AUC value (Figure 7).

Sage and thyme were the least effective Lamiaceae herbs in terms of their ability to increase nitric oxide. Treatment with sage at a concentration of 0.1% (w/v) resulted in total nitric oxide levels of 290.3 µmol/L/mg of protein and 343.1 µmol/L/mg of protein on day 2 and day 6 respectively. On the 2nd day of treatment the production of nitric oxide decreased significantly (p=0.0013); however, increased significantly (p=0.0001) on day 6 when compared to controls (Table 8). In worms feeding on LGM with sage at 0.5% (w/v), produced nitric oxide values of 387.4 µmol/L/mg of protein and 439.0 µmol/L/mg of protein were observed on day 2 and day 6 respectively, with a significant

(p=0.0002) increase in nitric oxide on the 6th day of treatment in comparison to controls (Table 8). It was noted that at 0.1% (w/v) nitric oxide levels were significantly (p=0.0095) lower than at 0.5% (w/v) over the span of 6 days (Figure 7). Thyme was the least effective in its ability to stimulate nitric oxide production over 6 days of treatment. At 0.1% (w/v), total nitric oxide levels were 264.5 µmol/L/mg of protein and 319.7 µmol/L/mg of protein on day 2 and day 6 respectively, which resulted in a significant (p=0.0007) decrease in the amount of nitric oxide on day 2 and significant (p=0.0000) increase on day 6 in comparison to controls (Table 8). At the thyme concentration of 0.5% (w/v), the nitric oxide formation was 368.6 µmol/L/mg of protein on day 2 and 337.5 µmol/L/mg of protein on day 6. On the 6th day of treatment there was significantly (p=0.0004) higher levels of nitric oxide formed when compared to controls (Table 8). Thyme at a concentration of 0.1% (w/v) was significantly (p=0.0132) less effective in raising nitric oxide levels in comparison to 0.5% (w/v) throughout the course of treatment as noted from AUC values (Figure 7).

Table 8: Effect of Lamiaceae Herbs on Levels of Nitric Oxide (μmol/L/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Nitric oxide (μmol/L/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil	0.1	310.1	0.0023#	373.0	0.0006*
	0.5	294.0	0.0006#	299.0	0.0000*
Oregano	0.1	264.3	0.0046#	408.4	0.0000*
	0.5	269.9	0.0068#	311.0	0.0000*
Rosemary	0.1	358.6	0.3442	333.8	0.0002*
	0.5	286.4	0.0948	203.9	0.1462
Sage	0.1	290.3	0.0013#	343.1	0.0001*
	0.5	387.4	0.1246	439.0	0.0002*
Thyme	0.1	264.5	0.0007#	319.7	0.0000*
	0.5	368.6	0.4587	337.5	0.0004*
Control	LGM	357.0	-	196.9	-

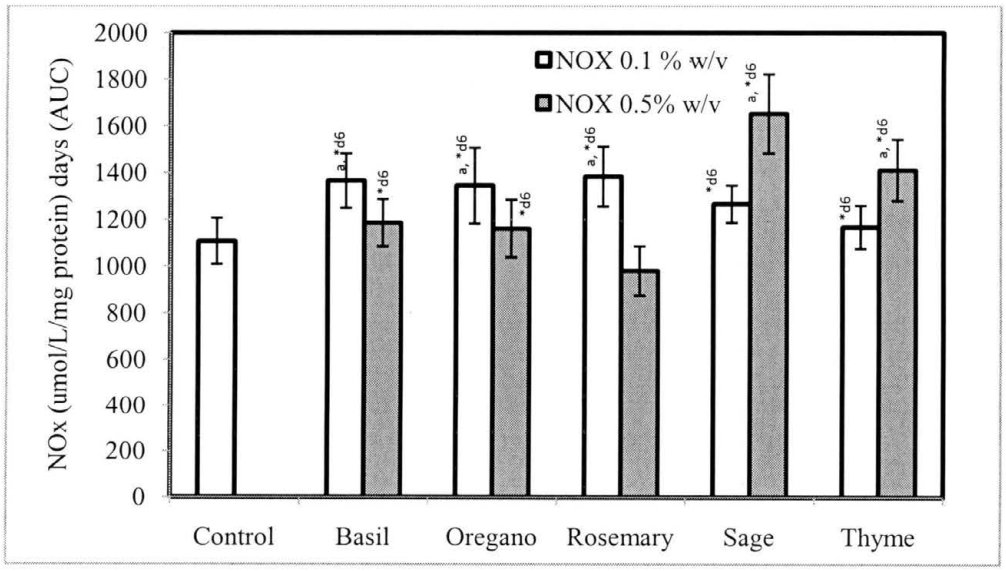

Figure 7: Overall Effectiveness of Different Lamiaceae Herbs on Total Nitric Oxide Levels Over 6 Days
a- indicates the treatment concentration which was significantly different ($p<0.05$)
*- indicates significant difference from the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Discussion

Molecular oxygen, being a diradical, has a high affinity for electrons and through evolutionary processes it has become an integral part of metabolism for some organisms. This inorganic compound provides a large thermodynamic driving force for the generation of adenosine triphosphate (ATP) via its role as the ultimate electron acceptor in the electron transport chain (ETC) (Berg et al., 2002). Cytochrome c oxidase in Complex IV of the ETC efficiently transfers four electrons to molecular oxygen and creates two molecules of water (Hill, 1994). However, there are instances in this enzymatic process in which there is a one electron reduction of molecular oxygen and highly reactive intermediates are formed such as the superoxide anion (0_2^-) (Alberts, 2004). Along with cytochrome c oxidase, other proteins that reduce molecular oxygen are for the most part quite efficient at not generating these highly reactive intermediates; however, these molecules are unavoidably produced and sometimes can diffuse out of the microenvironment (Berg et al., 2002). Superoxide, hydrogen peroxide, and species derived from these molecules are commonly referred to as reactive oxygen species (ROS) (Apel and Hirt, 2004). ROS are derivatives of oxygen that are in a more reactive state than molecular oxygen and the term is applied to free radicals and non-radical molecules, and can also include reactive nitrogen and chlorine species (Karihtala and Soini, 2007). Free radicals have been defined as any chemical species that have the capability of an independent existence, and these molecules are characterized by having one or more unpaired electrons (Tsukahara, 2007). Though the reasons for the deliberate production of ROS in cellular processes has been a topic of debate for well over 20 years, it is now well understood that ROS produced under normal physiological conditions have various

functions such as inducing apoptosis or necrosis, inducing or suppressing gene expression, and activating cell signaling cascades of which effect cell growth, division, and survival (Hancock et al., 2001). However, because of high reactivity the role of ROS can be paradoxical as they can oxidize macromolecules and impede normal cellular function. Therefore, many organisms have evolved different humoral and cellular mechanisms to manage oxidative injury and maintain redox homeostasis (Zhu et al., 2009). Of these, small antioxidant molecules and enzymes play a key role (Sies, 1997). However, when produced in excess these highly reactive molecules can overwhelm a cell's antioxidant defense and initiate lipid peroxidation, protein oxidation, as well as oxidize nucleic acids and therefore disrupt intracellular processes (Edeas and McCord, 2005). Excessive production of ROS and that can lead to an imbalance in the cellular redox homeostasis is commonly referred to as oxidative stress. Oxidative stress has long been suspected to play an important role in the pathogenesis of many diseases. Research over the past few decades has shown that diabetes, cancer, neurodegenerative diseases, infertility, and CVD are intimately linked with oxidative stress (Jakus, 2000; Madamanchi et al., 2005; Lin and Beal, 2006; Federico et al., 2007; Tremellen, 2008). Therefore reducing processes that generate ROS and/or modulating cellular antioxidant defenses may prove to be an effective strategy for preventing or managing these diseases.

Emerging evidence from epidemiological studies has suggested that plant based diets rich in foods such as fruits, vegetables, herbs, spices, and nuts are valuable in disease prevention and treatment (van't Veer et al., 2000; Kris-Etherton et al., 2002; Heber, 2004; Reddy and Katan, 2004). These foods are abundant sources of plant secondary metabolites such as polyphenols and carotenoids. These phytochemicals, due

to their chemical structures, can function as effective antioxidants (Scalbert and Williamson, 2000). Lamiaceae herbs, like many other herbs, are historically well known for their health benefits. It is now known that these herbs contain substantial amounts of phenolic compounds such as rosmarinic acid, caffeic acid, vanillic acid, and ferulic acid that may contribute to their health promoting properties (Kivilompolo and Hyotylainen, 2007).

In the current study, five Lamiaceae herbs were screened and included basil, oregano, rosemary, sage, and thyme and evaluated their ability to modulate antioxidant enzyme activity, nitric oxide biomarkers, and markers for oxidative induced damages *in vivo* in *Lumbricus terrestris*. This organism was allowed to feed ad libidum on a diet supplemented with Lamiaceae herbs, and biochemical tests were performed in seminal vesicles tissues. Seminal vesicles were selected as the target organ of study as this environment is especially sensitive to oxidative stress and it participates in many ROS mediated processes such as spermatogenesis and sperm development and maturation (Baker and Aitken, 2005). The results of this study indicated a modulatory effect of Lamiaceae herb treatment on two important antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). Upon *in vivo* treatment with Lamiaceae herbs at concentrations of 0.1% (w/v) and 0.5% (w/v), an overall 31.73% increase in superoxide dismutase activity (SOD) and 3.97% increase in catalase activity were observed when compared to basal levels in control worms (Table 8). Rosemary was most effective at increasing SOD activity with a 48.92% increase when compared to controls. Basil, sage, thyme, and oregano were also able to increase SOD activity (Table 8). Previous studies have also shown inductions of endogenous antioxidant enzyme activity upon

consumption of various dietary and medicinal plants. For example, human subjects taking a supplement composed of milk thistle, ginseng, turmeric, and green tea had 30% increases in SOD activity and a 54% increase in catalase activity in erythrocytes after 120 days of supplementation (Nelson et al., 2006). SOD, catalase, and glutathione peroxidase (GPx)/glutathione are the predominate antioxidants which are involved in the metabolism of superoxide and hydrogen peroxide (Zhu et al , 2009). The SOD enzymes efficiently dismutate superoxide anions into hydrogen peroxide and molecular oxygen where as catalase and GPx are involved in the stoichiometric conversion of hydrogen peroxide into water (Edeas and McCord, 2005). Although SOD, catalase, and GPx are all antoxidant enzymes they each are regulated differently in cells. sod-1 and sod-2 genes express various isoforms of SOD. The main isoforms are Mn-SOD (mitochondrial), Cu/Zn-SOD (cytoplasmic), and EC-SOD (extracellular) and they all are under the regulation of a promoter that can be recognized by a variety of transcription factors including SP-1/SP-3, AP-1/AP-2, C/EBP and NFκB. The transcription factors SP1 and SP3 as well as AP-1/AP-2 are activated through phosphorylation that is mediated either by mitogen activated protein kinase/ extracellular-signal-regulated-kinase (MAPK/ERK) signaling or via an atypical protein kinase c (Minc et al., 1999; Tanaka et al , 2000). The activation of these pathways is initiated through the binding of cytokines or insulin like growth factor (IGF) to insulin receptors which activates tyrosine kinase mediated phosphorylation (Samson and Wong, 2002). NFκB activation is initiated by phosphorylation mediated by TRAF2 and NIK. Phosphorylated NFκB is then able to translocate to the nucleus of the cell where is can bind to the promoter region of the SOD gene, thereby inducing its expression (Guo et al., 2003). Also the production of hydrogen peroxide by the increased

activity of SOD can independently relieve the inhibition of NFκB and facilitate in its nuclear translocation and subsequently increase the expression of SOD in a feed forward manner (Das et al., 1995). Moreover, NFκB can bind to the promoter region of catalase and other inflammatory factors and induce their expression. Glutathione peroxidase, the enzyme which requires glutathione to eliminate hydrogen peroxide, on the other hand is under the transcriptional regulation of the Antioxidant Response Element (ARE). Genes under the control of ARE are activated by the transcription factor, Nrf2, which also regulates the expression of other antioxidant enzymes such as glutathione s-transferase (GST), NOQ1, and glutathione reductase (GR), and cysteine transferase (Kang et al., 2005; Katsuoka et al., 2005). Phenolic compounds in natural products are known to modulate the MAPK pathway that activate ERK-1 and therefore activate SP1 and increase expression of SOD, independent of the tumor necrosis factor alpha (TNFα) and NFκB pathway (Yeh and Yen, 2006). Additionally many synthetic and natural phenolic compounds are known to activate the Nrf2 transcription factor, promote its nuclear translocation and therefore facilitate in the expression of genes under the regulation of ARE (Jaiswal, 2004; Nguyen et al., 2004; Banning et al., 2005). The apparent non- NFκB mediated increased expression of SOD via SP-1 and SP-3, and ARE mediated expression of GPx may contribute to lower increases of catalase activity even though there are increases in SOD expression (Hayakawa et al., 2003; Zelko et al., 2008). The hydrogen peroxide formed, which may have released NFκB from IκB, is rapidly removed by the GPx enzyme systems. This may also be indirectly observed by the overall 4.5% decrease in glutathione levels in herb treated worms, suggesting a very rapid utilization of GSH perhaps by GPx. Previous studies have shown glutathione peroxidase to be abundant in

the testis, epididymis, and spermatozoa with levels peaking during the time of puberty and spermatogenesis (Maiorino and Ursini, 2002). With the noted induction of SOD and antioxidant activity in seminal vesicle tissues, treatment with antioxidant rich herbs could possibly render these tissues more resistant to oxidative stress, and therefore facilitate healthy sperm development and maturation. In fact previous studies in rats and humans have shown protective benefits associated with antioxidant treatment with regards to sperm quality and count. In one such study rats with sodium nitrate induced oxidative stress had significantly lower sperm counts when compared to controls, however upon treatment with vitamins C and E the sperm counts were restored to basal levels (Yarube et al , 2009). In infertile men treated with the natural antioxidant, astaxanthin, levels of ROS and Inhibin B were significantly decreased while pregnancy rates were signifcanly higher than placebo groups (Comhaire et al., 2005). Spermatozoa are particularly vulnerable to oxidative stress because of their cellular organization. Earthworm spermatozoa as well as human spermatozoa undergo a nuclear and cytoplasmic extrusion process during maturation (Anderson et al., 1967; Cocuzza et al , 2007). The cytoplasm is a major source of enzymatic and chemical antioxidants. Therefore, the natural process of sperm development and maturation renders the sperm vulnerable to oxidative insult (Cocuzza et al., 2007). During spermatogenesis the sperm are not in contact with the antioxidant rich seminal plasma, therefore they rely strictly on the antioxidants in the tissues in which they are stored (Tremellen, 2008). Therefore, increasing antioxidant enzyme levels in these tissues may enhance protection and survival of the spermatozoa. In addition to testes, other tissues of the body can also confer protection from cellular damage. We have previously shown that *Lumbricus terrestris* treated with various herbs,

fruits, and spices were able to manage an acute hydrogen peroxide initiated oxidative stress in muscle tissues by inducing an antioxidant enzyme response (Hutton et al., 2009 and Thesis). An increase in superoxide dismutase and antioxidant activity in seminal vesicle tissue by Lamiaceae herb treatment may elucidate an important pathway for protecting developing spermatozoa against oxidative induced damages. The molecular mechanisms of functionality and effect on critical signaling pathways necessary for activation of SOD, GPx, and other antioxidant defenses form an important component of the next phase of investigation. Therefore, investigations into the molecular mechanisms of antioxidant protection mediated by Lamiaceae herbs were conducted using transgenic strains of C elegans expressing GFP tagged proteins of interest.

The increased antioxidant enzyme activity observed in Lamiaceae herb treated earthworms was accompanied by decreased levels of lipid peroxidation in seminal vesicles and reductions in morphological sperm deformations. Lipids are one of the most susceptible macromolecules to oxidative damage, and spermatozoa have a plasma membrane rich in polyunsaturated fatty acids (Baker and Aitken, 2005). Evidence from several studies link lipid peroxidation and the by-products of this reactions such as MDA and 4-hydroxynonenal (4-HNE) to various diseases such as atherosclerosis, type 2 diabetes, and cancer (Niki et al., 2005, Frederico et al., 2007). In the current study, Lamiaceae herb treatment lead to an overall 73.29% reduction in malondialdehyde levels when compared to controls (Table 8). Similar results were seen in another study with aqueous extracts of different Lamiaceae herbs (Dorman et al., 2003). It was shown that these herbs protect low density lipoprotein (LDL) particles from copper-induced oxidation with the greatest protection coming from rosemary and sage treatments. In our

study it was noted that treatment with rosemary reduced MDA levels by 86.04% when compared to controls. All Lamiceae herbs were able to lower MDA levels to some extent, with basil having the lowest effect with a 56.36% decrease in levels of MDA in comparison to controls (Table 8). Previous studies have also demonstrated that increased levels of MDA are associated with impairment in sperm motility and infertility (Agarwal, 2004). One such study indicated that upon exposure to various concentrations of hydrogen peroxide the number of sperm with morphological changes such as head abnormalities, nuclear fragmentation, and chromatin dispersion increased in a concentration dependent manner (Sanchez et al., 2006). Along with decreased MDA levels, the results from this study indicated Lamiaceae herb treatment taken as a whole resulted in a 36.65% decrease in sperm deformations in comparison to worms only feeding on lumbricus growth medium (Table 8). Treatment with thyme reduced deformations by 52.10% relative to controls and this was followed by rosemary, basil, oregano, and sage (Table 8). Along with these protective benefits associated with Lamiaceae treatment, there were minimal changes in DNA fragmentation when compared to control worms. There was a slight 0.75% increase in fragmentation of DNA observed in herb treatment groups (Table 8). A similar study conducted with sage, oregano, and rosemary showed that these herbs protected CaCo-2 cells from hydrogen peroxide induced DNA damage (Aherne et al., 2007). This study had similar results, where it was found that oregano reduced DNA fragmentation by 10.98%, and was followed by rosemary which reduced DNA fragmentation by 6.64% (Table 8).

A paradox exists in the functions of nitric oxide in different pathological and physiological processes. Biologically, NO forms many radicals such as nitrous oxide,

peroxynitrite, and peroxynitrous acid of which can oxidize various cellular components (Tremellen, 2008). Peroxynitrite induced oxidation has been linked with pathologies in diabetes and neurodegenerative disorders as well such as Alzheimer's disease (Smith et al., 1997; Chi et al., 2005; Pacher et al., 2007) In reproductive biology, some studies that suggest that high levels of nitric oxide can be deleterious to spermatozoa's kinetic characteristics (Balercia et al., 2004). Alerternatively, nitric oxide can function as a signaling molecule in various processes such as regulation of vascular tone, neurotransmission, and reproductive functions (Balercia et al., 2004). These processes are primarily mediated by activation of guanylyl cyclase dependent pathways (Moncada and Bolanos, 2006). When examining the detrimental effects of NO/ nitric oxide synthase (NOS) in male reproductive biology, the literature points to leukocytes in the testes region as the primary source of inducible nitric oxide synthase (iNOS). However male reproductive tissue especially germ and sertoli cells produce endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) which are under transcriptional regulation of AP1 and AP2. The NO formed as a result of eNOS expression is proposed to play an important role in junction restructuring and differentiation of germ cells into sperm cells. Extensive junction restructuring is required to assist germ cell movement from the basal compartment to the apical compartment of seminiferous tubules (Lee and Cheng, 2004). Decreased activation of nNOS and eNOS has been shown to result in a loss of germ cells from the seminiferous epithelium in testes. As previously described, plant phenolic compounds are known to modulate the MAPK/ERK pathway resulting in activation of AP1 and AP2. The observed overall 16.66% increase in the nitric oxide metabolites in the seminal vesicle could possibly

indicate an increased expression of eNOS and nNOS mediated by AP1 or AP2 transcription factors (Table 9). This coupled with elevated antioxidant gene expression may have contributed to increased spermatogenesis and enhanced AOX protection as evidenced by lower oxidative stress markers and superior sperm quality.

Table 9: The Effect of Different Lamiaceae Herbs on Antioxidant Enzyme Activity, Markers of Oxidative Stress and Nitric Oxide Biomarkers Relative to Controls.

SOD- Superoxide Dismutase, CAT- Catalase, GLU- Reduced Glutathione, MDA- Malondialdehye, DFO- Sperm Deformity, DFR- DNA Fragmentation, NOX- Nitric Oxide

	Percent Change from Control Values (%)						
Herbs	**SOD**	**CAT**	**GLU**	**MDA**	**DFO**	**DFR**	**NOX**
Basil	34.55	21.21	-11.39	-56.36	-44.40	4.01	15.18
Oregano	15.44	-0.87	15 21	-75 16	-20 90	-10.98	13 15
Rosemary	48.92	-7.39	-18.01	-86 04	-47 63	-6.65	6 74
Sage	30.05	-2.89	-3 59	-75.55	-18.22	6 65	31.76
Thyme	29.69	9 79	-4.81	-73 34	-52.10	10 69	16.46
AVG of all herbs	**31.73**	**3.97**	**-4.52**	**-73.29**	**-36.65**	**0.75**	**16.66**

When nitric oxide metabolite levels (NOX) in tissues of the seminal vesicles were measured after supplementation with Lamiaceae herbs, treatment with sage resulted in the highest NOX levels, with 31.76% increase when compared to controls. Treatment with thyme, basil, and oregano also increased levels of NOX and treatment rosemary resulted in a 6.74% increase in NOX (Table 9). Previous studies on herbs from the Lamiaceae family such as *Salvia miltiorrhiza*, have also increased levels of eNOS mRNA ,eNOS, and NO in human umbilical vein endothelial cells (Steinkamp-Fenske et al., 2007). In this study the increase in NOX upon treatment with Lamiaceae herbs occurred with concurrent increases in antioxidant enzyme activity and a decrease in lipid peroxidation and sperm deformity. This increased production of nitric oxide without an

increase in oxidative damage may suggest a unique NOX mediated mechanism of action by Lamiaceae phytochemicals and needs further investigation.

The current study has also revealed that herbs from the same family can have different effects on the redox status of tissues. These differences could possibly be attributed to the variation in both phytochemical content and profile among plants which belong to the same family and that share some bioactive compounds (Kivilompolo and Hyotylainen, 2007).

CHAPTER II

THE PHYSIOLOGICAL EFFECTS OF DIFFERENT LAMIACEAE HERBS ON REDOX AND NITRIC OXIDE SIGNALING IN SEMINAL VESICLES OF HYDROGEN PEROXIDE STRESSED *LUMBRICUS TERRESTRIS*

Hydrogen Peroxide Induced Oxidative Stress

Hydrogen peroxide is one of the major reactive oxygen species (ROS) produced by living tissues (Gao et al., 2001). The reactivity of this molecule is low, enabling it to diffuse through membranes where it can participate in the Fenton reaction with metals such as iron to generate the highly reactive hydroxyl radical (Valko et al., 2007). Therefore, uncontrolled production of hydrogen peroxide can lead to oxidative stress. Frequently hydrogen peroxide is used in laboratory settings as a means to induce oxidative damages in tissues and cells (Wei et al., 2000; Gao et al., 2001). This allows one to monitor the physiological and genetic changes in response to a state of oxidative stress. Additionally, this enables researchers to evaluate the effectiveness of various treatments in restoring redox homeostatis (Hsieh et al., 2004; Heo and Lee, 2004).

Recently our lab has shown that the addition of 12mM hydrogen peroxide to standard LGM can induce oxidative damages within the muscle tissues of *Lumbricus terrestris* (Hutton et al., 2009). In the same study we revealed that fruit and spice extracts were able to manage the acute oxidative stress through induction of an antioxidant enzyme response.

Objective of Study

The objective of the current study was to determine the modulatory effect of Lamiaceae herbs on hydrogen peroxide induced oxidative stress in seminal vesicles of *Lumbricus terrestris*. Specifically, the effect of Lamiaceae phytochemicals on redox status, antioxidant enzymes (superoxide dismutase, catalase), oxidative damage to DNA, and NO mediated spermatogenesis.

Materials and Methods

Preparation of Media

In this study, materials and methodology were the same as previously described in Chapter I (page 14). Based on results from Chapter I, it was determined that treatment with Lamiaceae herbs at a concentration of 0.1% (w/v) was most effective in terms of modulating redox homeostasis. Additionally, it was observed that the most effective herbs were basil, oregano, and thyme. Therefore in this study, treatment plates contained concentrations of 0.1% w/v of the top 3 Lamiaceae herbs and 12mM hydrogen peroxide added to the standard LGM petri plate. Also, a LGM + 12mM hydrogen peroxide treatment served as the negative control.

Results

Sperm Deformations

In this study, the top 3 Lamiceae herbs from Chapter I were evaluated for their effectiveness at managing various endpoints associated with hydrogen peroxide induced oxidative stress. *Lumbricus terrestris* only consuming lumbricus growth medium (LGM) for 6 days were established as controls in this study. After feeding for two days ad

libitum, 22.8% of spermatozoa exhibited a deformation. On day 6, 23.75% of all spermatozoa evaluated had some type of deformation in control worms (Table 10).

Upon the addition of 12mM hydrogen peroxide (PER) to the standard LGM, significant increases in sperm deformations were observed over the duration of 6 days. On day 2, 24.0% of spermatozoa exhibited a morphological deformation (Table 10). On the sixth day, 36.7% of sperm were deformed which was significantly (p=0.0038) higher than controls (Table 10). The peroxide treated worms had a significantly (p=0.0095) increased ratio of deformed spermatozoa to normal spermatozoa when compared to controls based on the calculations of the AUC (Figure 8).

L. terrestris treated with 0.1% (w/v) thyme (TH) + PER exhibited lower levels of sperm morphological deformations than worms treated only with 12mM peroxide. On day 2 and 6, deformities were calculated to be 20.3% and 18.3% respectively, which was significantly (p=0.0276; p=0.0000) lowered when compared to the 12mM peroxide group (Table 10). Upon observation of the total effectiveness of treatment, it was revealed that thyme +12mM peroxide significantly (p=0.0056) decreased sperm deformations in comparison to the 12mM peroxide treatment (Figure 8). When treatment with thyme + peroxide was compared to control values, there was no significant difference observed on day 2. However on day 6, the treatment had significantly (p=0.0109) lower levels of sperm deformations that controls (Table 10). Over the duration of the six day treatment, it was observed that treatment with thyme + peroxide had significantly (p=0.0136) less sperm deformations than controls (Figure 8).

Treatment with 0.1% (w/v) oregano (OR) + 12mM peroxide also significantly decreased morphological sperm deformations. On the second day of treatment, 22.9% of

all sperm observed had a deformity (Table 10). The percentage of deformations decreased to 19.8% on day 6 which was significantly (p=0.0038) lower than the peroxide group on the sixth day (Table 10). Based on calculations of the AUC, the oregano + 12mM peroxide treatment group had significantly (p=0.0072) lower levels of sperm deformations than the treatment which only had 12mM peroxide (Figure 8). When compared to control values, treatment with oregano + peroxide was not significantly different on day 2, however on day 6 levels of sperm deformations were significantly (p=0.0457) lower (Table 10). Based on calculations of the AUC, treatment with oregano + peroxide had significantly (p=0.0298) lower levels of sperm deformities than controls (Figure 8).

Treatment with 0.1% (w/v) basil (BA) + 12mM peroxide resulted in significant changes in sperm morphology as well. On day 2, 29.3% of spermatozoa had deformations which was significantly (p=0.0393) higher than the group treated with only 12mM peroxide (Table 16). However, on the sixth day the number of deformations decreased to 21.1% which was significantly (p=0.0043) lower than the 12mM peroxide group (Table 10). It was observed that over the duration of treatment, the worms treated with BA + PER had significantly (p= 0.0134) lower percentages of sperm deformations than the 12mM peroxide group (Figure 8). Treatment with basil + peroxide significantly (p=0.0176) increased deformations on day 2 when compared to controls, but on day 6 there was not a significant difference between treatments and controls (Table 10). When the AUC was calculated, it was observed that the controls had significantly (p=0.0316) lower levels of sperm deformations than the treatment with basil + peroxide (Figure 8).

Table 10: Effect of Lamiaceae Herb Treatment on Sperm Deformations in Peroxide Stressed *L. terrestris*

* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)

†* indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

		Sperm Deformations (%)					
Concentration % (w/v)		Day 2	*P* value (T+P vsC)	*P* value (T+P vs P)	Day 6	*P* value (T+P vsC)	*P* value (T+P vs P)
Basil + Peroxide	0.1	29.3	0.0176#	0.0393#	21.1	0.0602	0.0043*
Oregano+ Peroxide	0.1	22.9	0.2802	0.2521	19.8	0.0457*	0.0038*
Thyme + Peroxide	0.1	20.3	0.4167	0.0276*	18.3	0.0109*	0.0000*
Peroxide	12mM H202 + LGM	24.0	0.2104		36.7	0.0038†#	
Control	LGM	22.8		-	23.75		-

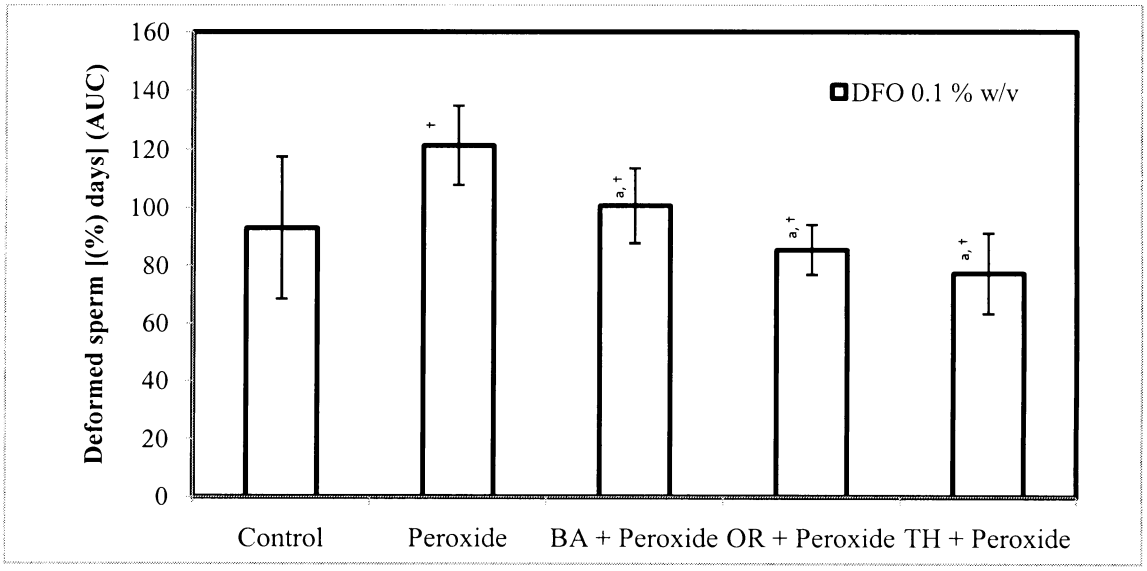

Figure 8: Overall Effectiveness of Lamiaceae Herb Treatment on Sperm Deformations (DFO) Over the Duration of 6 Days in Peroxide Stressed *L. terrestris*.

a- indicates significant difference between Treatment+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)

†- indicates significant difference from the control (AUC) ($p<0.05$)

The data is represented as means ± SEM

Malondialdehyde Assay

The extent of lipid oxidation in seminal vesicles was measured by TBARS assay to assess the amount of malondialdehyde formed. In control worms that fed on LGM the levels of MDA after 2 days was 0.6 mmol/mg of protein. On the 6^{th} day, the level of MDA decreased to 0.4 mmol/mg of protein (Table 11).

12mM hydrogen peroxide was added to the LGM, and significant increases in MDA levels were observed. On day 2 and day 6, the levels of MDA were 0.7 mmol/mg of protein and 0.8 mmol/mg of protein respectively, which was significantly (p=0.0006; p=0.0003) higher than controls (Table 11). Over the duration of treatment, *L. terrestris* which fed only on LGM had significantly (p=0.0026) lower levels of MDA than 12mM peroxide + LGM based on calculations of the AUC (Figure 9).

The addition of Lamiaceae herbs at a concentration of 0.1% (w/v) to LGM containing 12mM hydrogen peroxide significantly lowered levels of lipid oxidation. Oregano + 12mM peroxide was most effective at decreasing MDA. On day 2, the levels of MDA were 0.4 mmol/mg of protein which was significantly (p=0.0000) decreased when compared to worms which only fed on 12mM peroxide (Table 11). On the sixth day of treatment, the lipid oxidation values were 0.1 mmol/mg of protein which was also significantly (p=0.0002) lower than the 12mM peroxide treatment (Table 11). It was observed that the OR + PER treatment had significantly (p=0.0025) decreased MDA levels when compared to the 12mM peroxide treatment throughout the six days of exposure (Figure 9). When treatment with oregano + peroxide was compared with controls, MDA values were significantly (p=0.0280) higher on day 2 (Table 11). However on day 6, there was no significant difference between treatment and controls

(Table 11). Over the duration of 6 days, the controls had significantly (p=0.0349) lower levels of MDA than treatment with oregano + peroxide (Figure 9).

Treatment with 0.1% (w/v) TH + PER also resulted in decreased lipid oxidation when compared to MDA levels in worms treated with 12mM peroxide alone. Malondialdehyde levels were 0.5 mmol/mg of protein and 0.2 mmol/mg of protein on day 2 and day 6 respectively in thyme + peroxide treated worms, which were significantly (p=0.0233; p=0.0009) lower than 12mM peroxide treatments (Table 11). Over the 6 day treatment, TH + PER MDA levels were significantly (p=0.0032) lower than 12mM peroxide alone as indicated by the AUC (Figure 9). The treatment with thyme + peroxide was not significantly different from controls on day 2. However on day 6, lipid oxidation values were significantly (p=0.0479) higher in seminal vesicles of thyme + peroxide treated worms than controls (Table 11). Over the course of the six day treatment, it was observed that MDA levels were significantly (p=0.0129) higher in thyme + peroxide treatments than controls which only consumed LGM (Figure 9).

L. terrestris which consumed 0.1% (w/v) BA + PER had decreased MDA levels when compared to worms which only consumed 12mM peroxide. On the second day of treatment, lipid oxidation values were 0.4 mmol/mg of protein, which was significantly (p=0.0003) decreased in comparison to the peroxide treatment (Table 11). The MDA values on day 6 were 0.2 mmol/mg of protein, which was significantly (p=0.0030) lower than the sixth day of 12mM peroxide treatment (Table 11). Over the span of 6 days, worms which fed on BA + PER had significantly (p=0.0035) lower levels of MDA than the 12mM peroxide treated worms (Figure 9). The levels of lipid oxidation in basil + peroxide treated worms was significantly (p=0.0333; p=0.0453) higher than control

worms on day 2 and day 6, respectively (Table 11). Upon calculation of the AUC, the lipid oxidation levels were significantly (p=0.0108) lower in control worms than basil + peroxide treatments over the duration of six days (Figure 9).

Table 11: Effect of Lamiaceae Herbs on MDA (mmol/mg of protein) Formation in Seminal Vesicles of Peroxide Stressed *L. terrestris* as Measured by TBARS.
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†* indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

Concentration % (w/v)		MDA (mmol/mg of protein) Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	0.4	0.0333#	0.0003*	0.2	0.0453#	0.0030*
Oregano+ Peroxide	0.1	0.4	0.0280#	0.0000*	0.1	0.1959	0.0002*
Thyme + Peroxide	0.1	0.5	0.0531	0.0233*	0.2	0.0479#	0.0009*
Peroxide	12mM H202 + LGM	0.7	0.0006†#		0.8	0.0003†#	
Control	LGM	0.6		-	0.4		-

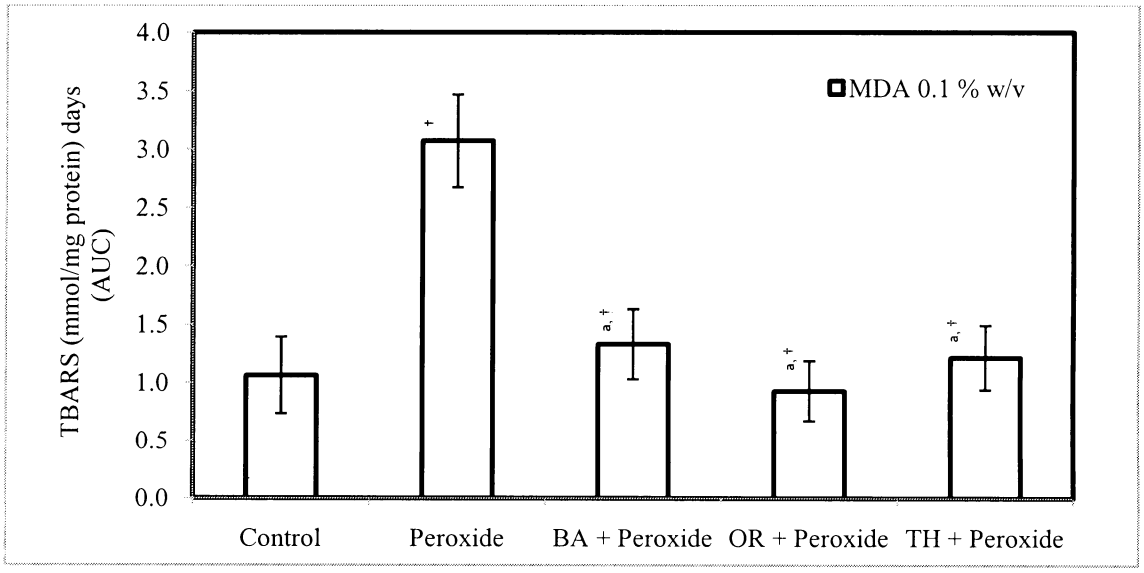

Figure 9: Overall Effectiveness of Lamiaceae Herbs on Levels of MDA in Seminal Vesicles of Peroxide Stressed *L. terrestris* Over 6 Days Measured as TBARS
a- indicates significant difference between Herb+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

DNA Fragmentation

In order to assess the extent of free radical induced damage to spermatozoa, the percentage of DNA fragmentation was measured using the DPA assay previous described. In control worms that fed on LGM, it was shown that on the second day of treatment the amount of DNA fragmentation was 46.0 %. On day 6, the extent of fragmentation decreased to 44.9% (Table 12).

The addition of 12mM hydrogen peroxide to LGM increased DNA fragmentation over the 6 days of treatment. The percentages of DNA fragmentation were 52.3% and 46.4% on day 2 and day 6 respectively, with a significant (p=0.0155) increase in comparison to controls on day 2 (Table 12). It was observed that the percentage of DNA fragmentation was significantly (p=0.0304) higher in worms which consumed 12Mm peroxide + LGM than worms which only consumed LGM over the duration of treatment (Figure 10).

Treatment with 0.1% (w/v) thyme + 12mM peroxide proved to be effective at decreasing DNA fragmentation. On the second day of treatment, the extent of fragmented DNA was 44.0%, which was significantly (p=0.0140) lower than peroxide group on day 2 (Table 12). On day 6, the percentage of DNA fragmentation was 46.4% (Table 12). Overall, TH + PER significantly (p=0.0420) decreased DNA strands breaks when compared to 12mM peroxide treatments as determined by the AUC (Figure 10). When comparisons were made between controls and thyme + peroxide, DNA fragmentation was significantly (p=0.0383) lower on day 2 and significantly (p=0.0179) higher on day 6 in the thyme + peroxide treatment (Table 12). However, when evaluating total

effectiveness of treatment over the duration of 6 days, there was no significant difference between controls and TH + PER (Figure 10).

Treatment with 0.1% (w/v) basil + 12mM peroxide was not effective at reducing levels of DNA fragmentation. On day 2 and 6, fragmentation in DNA was 48.5% and 45.5% (Table 12). It was concluded from AUC values that treatment with BA + PER was not significantly different than treatment with only 12mM peroxide (Figure 10). The levels of DNA fragmentation were similar among the basil + peroxide treatment and the controls, with no significant differences between treatments (Table 12). It was concluded from the calculation of the AUC that over the duration of six days there was not a significant difference in DNA fragmentation between the control worms and those treated with basil + peroxide (Figure 10).

Worms which consumed 0.1% (w/v) OR + PER did not show significant changes in the amount of DNA fragmentation as well. Tests conducted revealed fragmentations of 49.5% and 46.9% on day 2 and day 6 respectively (Table 12). The overall effectiveness of treatment revealed no significant difference between OR + PER treated *L. terrestris* and the 12mM peroxide treatment based on calculations of the AUC (Figure 10). When compared to controls, the levels of DNA fragmentation were not significantly different on day 2. However, on day 6 the levels of fragmented DNA was significantly (p=0.0191) higher in worms treated with oregano + 12mM peroxide than controls which consumed LGM (Table 12). It was observed that levels of DNA fragmentation were significantly (p=0.0420) higher in OR + PER treatments than controls over the duration of 6 days (Figure 10).

Table 12: Effect of Lamiaceae Herb Treatments on DNA Fragmentation (%) in Peroxide Stressed *Lumbricus terrestris*
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†∗ indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

Concentration % (w/v)		DNA Fragmentation (%)					
		Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	48.5	0.3821	0.0537	45.5	0.3474	0.4245
Oregano+ Peroxide	0.1	49.5	0.0817	0.1385	46.9	0.0191#	0.3155
Thyme + Peroxide	0.1	44.0	0.0383*	0.0140*	48.9	0.0179#	0.2512
Peroxide	12mM H202 + LGM	52.3	0.0155†#		46.4	0.4963	
Control	LGM	46.0		-	44.9		-

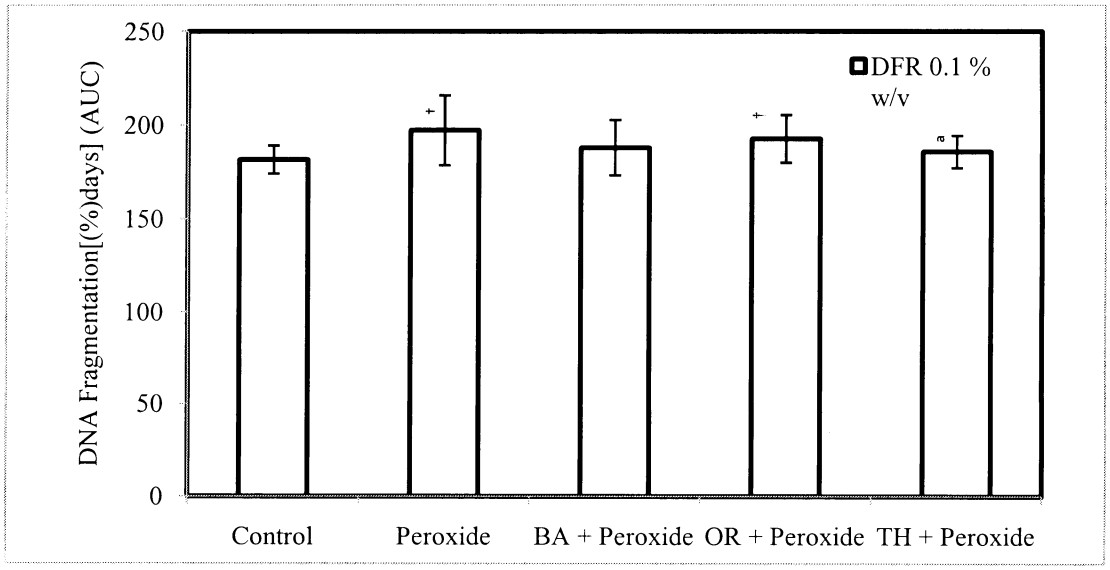

Figure 10: Overall Effectiveness of Lamiaceae Treatment on Levels of DNA Fragmentation (DFR) Over 6 Days in Peroxide Stressed *Lumbricus terrestris*
a- indicates significant difference between Herb+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

Superoxide Dismutase

The superoxide dismutase (SOD) activity was quantified using the NBT/ diformazan assay discussed previously. In control worms on day 2, the level of diformazan formed was 111.4 μmol/mg of protein. Tests on day 6 revealed that the levels of diformazan had decreased to 89.7 μmol/mg of protein (Table 13).

12mM peroxide added to LGM significantly lowered levels of diformazan over the duration of treatment. On day 2 and day 6, diformazan levels were 79.7 μmol/mg of protein and 84.7 μmol/mg of protein, which was significantly (p=0.0005; p=0.0044) decreased when compared to controls (Table 13). Upon determination of the AUC, it was observed that treatment with 12mM peroxide significantly (p=0.0030) lowered levels of diformazan (Figure 11) when compared with controls which only consumed LGM. The addition of 12mM peroxide induced SOD activity as indicated by lower levels of diformazan.

The overall effectiveness of 0.1% (w/v) basil + 12mM peroxide treatment on diformazan formation was not significantly different when compared to 12mM peroxide indicating no change in SOD activity. On day 2, diformazan levels were 82.7 μmol/mg of protein, which was significantly (p=0.0209) increased in comparison to 12mM peroxide (Table 13). On the sixth day of treatment, the level of diformazan was 83.2 μmol/mg of protein (Table 13). Calculation of the AUC revealed no significant differences between treatments over the span of 6 days (Figure 11). Comparison to control worms which only consumed LGM revealed significantly (p=0.0010; p=0.0420) lower levels of diformazan on day 2 and day 6, respectively (Table 13). It was observed that SOD activity was significantly (p=0.0131) higher over the duration of 6 days in the basil + peroxide

treatment group when compared to the controls as indicated by lower levels of diformazan (Figure 11).

Consumption of 0.1% (w/v) OR + PER increased levels of diformazan formation over the course of treatment. On day 2 and day 6, amounts of diformazan were 75.5 μmol/mg of protein and 118.4 μmol/mg of protein respectively, which was significantly (p=0.0217) higher on day 6 than 12mM peroxide treatments (Table 13). Overall, it was observed that OR + PER had significantly (p=0.0152) less SOD activity over the 6 day treatment than 12mM peroxide alone as indicated by the increased levels of diformazan (Figure 11). On the second day of treatment, diformazan levels were significantly (p=0.0006) lower in the oregano + peroxide treatment group than controls (Table 13). However on day 6, SOD activity was lower than controls as indicated by the significantly (p=0.0398) higher diformazan levels (Table 13). Over the span of 6 days, there was not a significant difference in SOD activity between the worms which consumed LGM and oregano + 12mM peroxide (Figure 11).

Similarly, treatment with 0.1% (w/v) TH + PER resulted in increases in the formation of diformazan. Levels of diformazan were 95.2 μmol/mg of protein and 100.8 μmol/mg of protein on day 2 and day 6 respectively, which were both significantly (p=0.0005; p=0.0044) higher than 12mM peroxide treatments (Table 13). Based on AUC values it was noted that treatment with TH + PER significantly (p=0.0142) increased diformazan formation when compared to 12mM peroxide treated worms (Figure 11). When comparisons to controls were made, diformazan levels in thyme + peroxide treatments were significantly (p=0.0181) lower on day 2 and significantly (p=0.0029) higher on day 6 (Table 13). Therefore, after calculation of the AUC it was observed that

there was not a significant difference in SOD activity between the controls and thyme + 12mM peroxide treatment groups (Figure 11).

Table 13: Effect of Lamiaceae Herbs on Superoxide Dismutase Activity as Indicated by Levels of Diformazan (μmol/mg of protein) in Peroxide Stressed *L. terrestris*
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†* indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

		Diformazan (μmol/mg of protein)					
Concentration % (w/v)		Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	82.7	0.0010*	0.0209#	83.2	0.0420*	0.1931
Oregano+ Peroxide	0.1	75.5	0.0006*	0.1905	118.4	0.0398#	0.0217#
Thyme + Peroxide	0.1	95.2	0.0181*	0.0005#	100.8	0.0029#	0.0033#
Peroxide	12mM H202 + LGM	79.7	0.0005†*		84.7	0.0044†*	
Control	LGM	111.4		-	89.7		-

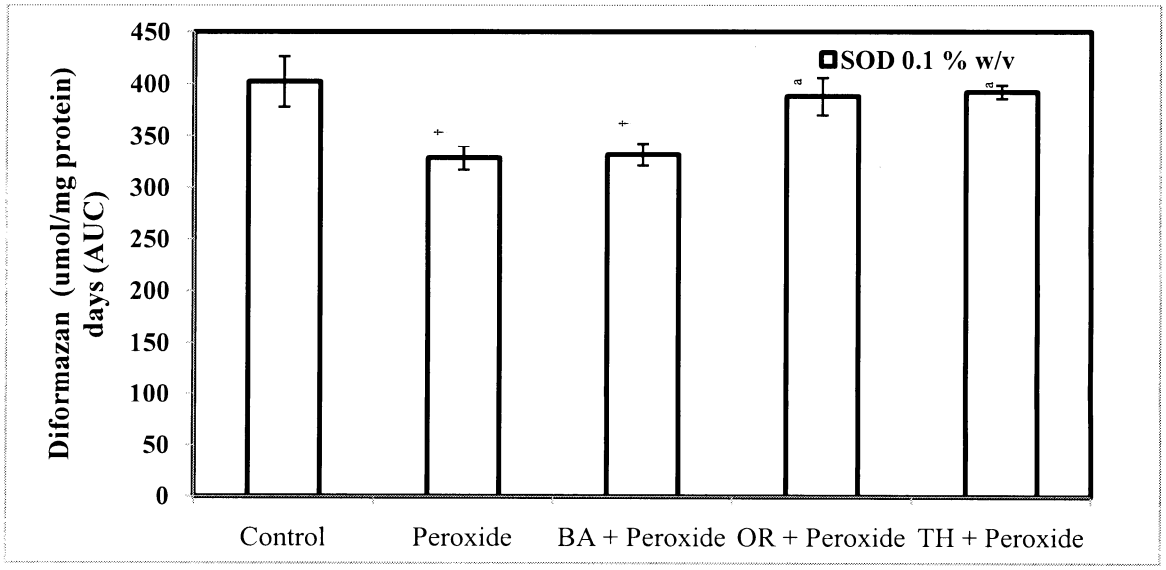

Figure 11: Overall Effectiveness of Lamiaceae Treatment on Superoxide Dismutase Activity as Indicated by Levels of Diformazan in Peroxide Stressed *L. terrestris* Over 6 Days
a- indicates significant difference between Treatment+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

Catalase

Removal of hydrogen peroxide, a powerful reactive oxygen species and product of SOD mediated reactions, requires the enzymatic action of catalase. In control worms which fed only on LGM, the catalase activity was 295.4 mU/ mg of protein after two days. The catalase activity after six days of treatment was 278.6 mU/mg of protein (Table 14).

12mM hydrogen peroxide + LGM lowered catalase activity on all days. On day 2 and day 6 the enzyme activity was 256.4 mU/mg of protein and 257.3 mU/mg of protein respectively, which was significantly (p=0.0000; p=0.0000) decreased in comparison to controls (Table 14). Upon calculation of the AUC, it was observed that over the duration of treatment, the controls had significantly (p=0.0226) higher catalase activity than 12mM peroxide treatments (Figure 12).

The addition of Lamiaceae herbs to 12mM peroxide treatments resulted in variable changes in catalase activity. 0.1% (w/v) BA + PER catalase activity was 207.0 mU/mg of protein on day 2, which was significantly (p=0.0118) lower than 12mM peroxide (Table 14). On the sixth day of treatment, enzyme activity increased to 251.4 mU/mg of protein which was significantly (p=0.0001) higher than the 12mM peroxide treatment (Table 14). However, when the overall effectiveness of treatment was calculated there was no significant difference between basil + 12mm peroxide and 12mM peroxide (Figure 12). On day 2 and day 6, catalase activity was significantly (p=0.0006; p=0.0442) lower in the basil + peroxide treatments when compared to controls (Table 13). Upon calculation of the AUC, it was observed that enzyme activity was significantly

(p=0.0196) higher in worms which consumed LGM than worms treated with BA + PER (Figure 12).

Lumbricus terrestris which consumed 0.1% (w/v) OR + PER had similar changes in catalase activity. On the second day, enzyme activity was 215.5 mU/mg of protein, which was significantly (p=0.0006) lower than 12mM peroxide treatments. Catalase activity was 261.3 mU/mg of protein on the sixth day, which was significantly (p=0.0005) higher than the 12mM peroxide group (Table 14). Based on calculations of the AUC, there was not a significant difference between OR + PER and the 12mM peroxide treatment over the span of 6 days (Figure 12). When compared to controls, catalase activity was significantly (p=0.0000) lower in the oregano + peroxide treatment (Table 14). However, on day 6 there was not a difference in enzyme activity between groups. Upon calculation of the overall effectiveness of treatment, it was observed that catalase activity was significantly (p=0.0201) higher in controls than the oregano + peroxide treatment over 6 days (Figure 12).

Similar to the previous Lamiaceae herbs, TH + PER treatment significantly modulated catalase activity. On day 2, catalase activity was 209.3 mU/mg of protein, which was significantly (p=0.0063) lower than the 12mM peroxide group (Table 14). On day 6, the enzyme activity increased to 246.4 mU/mg of protein, which was significantly (p=0.0032) higher than the 12mM peroxide treatment (Table 14). As was the case with the previous herbs, the overall effect of the TH + PER was not significantly different than treatment with 12mM peroxide (Figure 12). When compared to control values, catalase activity in worms treated with TH + PER was significantly (p=0.0003; p=0.0119) lower on day 2 and day 6, respectively (Table 14). Over the duration of treatment, it was

observed that catalase activity was significantly higher (p=0.0187) in the control group than the thyme + peroxide treatment based on calculation of the AUC (Figure 12).

Table 14: Effect of Lamiaceae Herbs on Catalase Activity (mU/mg of protein) in Peroxide Stressed *Lumbricus terrestris*
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†* indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

Concentration % (w/v)		Catalase (mU/mg of protein) Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	207.0	0.0006*	0.0118*	251.4	0.0442*	0.0001#
Oregano+ Peroxide	0.1	215.5	0.0000*	0.0006*	261.3	0.1052	0.0005#
Thyme + Peroxide	0.1	209.3	0.0003*	0.0063*	246.4	0.0119*	0.0032#
Peroxide	12mM H202 + LGM	256.4	0.0000†*		257.3	0.0000†*	
Control	LGM	295.4		-	278.6		-

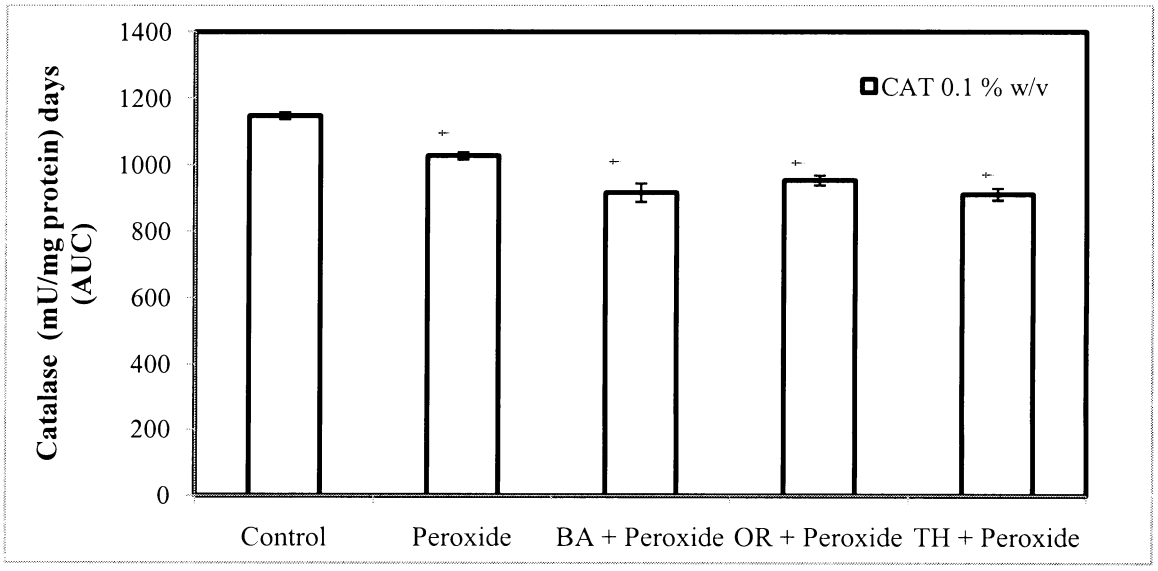

Figure 12: Overall Effectiveness of Lamiaceae Herbs on Catalase Activity Over 6 Days in Peroxide Stressed *L. terrestris* Over 6 Days
a- indicates significant difference between Treatment+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

Glutathione

Glutathione plays an important role in many biological processes and is a critical cellular antioxidant involved in maintaining cytosolic redox homeostasis. In control worms, on day 2 the level of glutathione was 72.2 mmol/mg of protein. On day 6 the amount of glutathione decreased to 67.2 mmol/mg of protein (Table 15).

The addition of 12mM peroxide to LGM resulted in significant changes in levels of glutathione. On day 2, glutathione levels were 70.2 mmol/mg of protein which was significantly (p=0.0044) lower than controls (Table 15). On the sixth day of treatment, levels of glutathione slightly increased to 70.3 mmol/mg of protein, which was significantly (p=0.0340) higher than controls on day 6 (Table 15). From AUC values it was noted that was treatment with 12mM peroxide did not significantly change levels of glutathione over the span of 6 days when compared to controls (Figure 13).

Treatment with 0.1% (w/v) BA + PER resulted in the most significant increases in glutathione in comparison to 12mM peroxide. Levels of glutathione were 76.6 mmol/mg of protein and 106.8 mmol/mg of protein on day 2 and day 6 respectively, which were both significantly (p=0.0022; p=0.0000) increased when compared to 12mM peroxide (Table 15). It was observed that BA + PER significantly (p=0.0095) increased glutathione levels in comparison to the 12mM peroxide treatment (Figure 13). When compared to controls, the glutathione levels in basil + 12mM peroxide were significantly (p=0.0112; p=0.0000) higher on day 2 and day 6, respectively (Table 15). Evaluation of the overall effectiveness of treatment revealed that worms treated with BA + PER had significantly (p=0.0092) higher levels of glutathione that worms which consumed LGM over the span of 6 days (Figure 13).

In *L. terrestris* which consumed 0.1% (w/v) thyme + 12mM peroxide also increased levels of glutathione were observed. On day 2 and 6 levels of glutathione were 84.0 mmol/mg of protein and 97.4 mmol/mg of protein respectively, which was significantly (p=0.0005; p=0.0004) higher than the 12mM peroxide treatments (Table 15). Upon calculations of the AUC, it was noted that treatment with TH + PER resulted in significantly (p=0.0098) higher glutathione levels than the 12mM peroxide group (Figure 13). Worms treated with thyme + peroxide had significantly (p=0.0004; p=0.0006) higher glutathione levels on day 2 and day 6, respectively than worms which consumed LGM on these days (Table 15). The AUC values indicated that over the duration of 6 days, *L. terrestris* treated with thyme + peroxide had significantly (p=0.0096) higher levels of glutathione than controls (Figure 13).

0.1% (w/v) oregano + 12mM peroxide did not have the same effect as the previous herbs on increasing glutathione levels when compared to the 12mM peroxide treatment. On day 2, glutathione levels were 74.4 mmol/mg of protein, which was significantly (p=0.0008) higher than the 12mM peroxide treatment (Table 15). On day 6, the glutathione levels decreased to 68.0 mmol/mg of protein (Table 15). The overall effectiveness of treatment with OR + PER was not significantly different than treatment with 12mM peroxide (Figure 13). On the second day of treatment, worms consuming OR + PER had significantly (p=0.0297) higher glutathione levels when compared to controls (Table 15). However, on the sixth day of treatment there was no difference between controls and OR + PER. Upon calculation of the AUC, there was not a significant difference in glutathione levels between the oregano + peroxide group and controls (Figure 13).

Table 15: Effect of Lamiaceae Herbs on Levels of Glutathione in the Tissues of Seminal Vesicles in Peroxide Stressed *Lumbricus terrestris*
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†* indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

Concentration % (w/v)		Glutathione (mmol/mg of protein) Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	76.6	$0.0112^{\#}$	$0.0022^{\#}$	106.8	$0.0000^{\#}$	$0.0000^{\#}$
Oregano+ Peroxide	0.1	74.4	$0.0297^{\#}$	$0.0008^{\#}$	68.0	0.1361	0.2560
Thyme + Peroxide	0.1	84.0	$0.0004^{\#}$	$0.0005^{\#}$	97.4	$0.0006^{\#}$	$0.0004^{\#}$
Peroxide	12mM H202 + LGM	70.2	$0.0044^{\dagger *}$		70.3	$0.0340^{\dagger \#}$	
Control	LGM	72.2		-	67.2		-

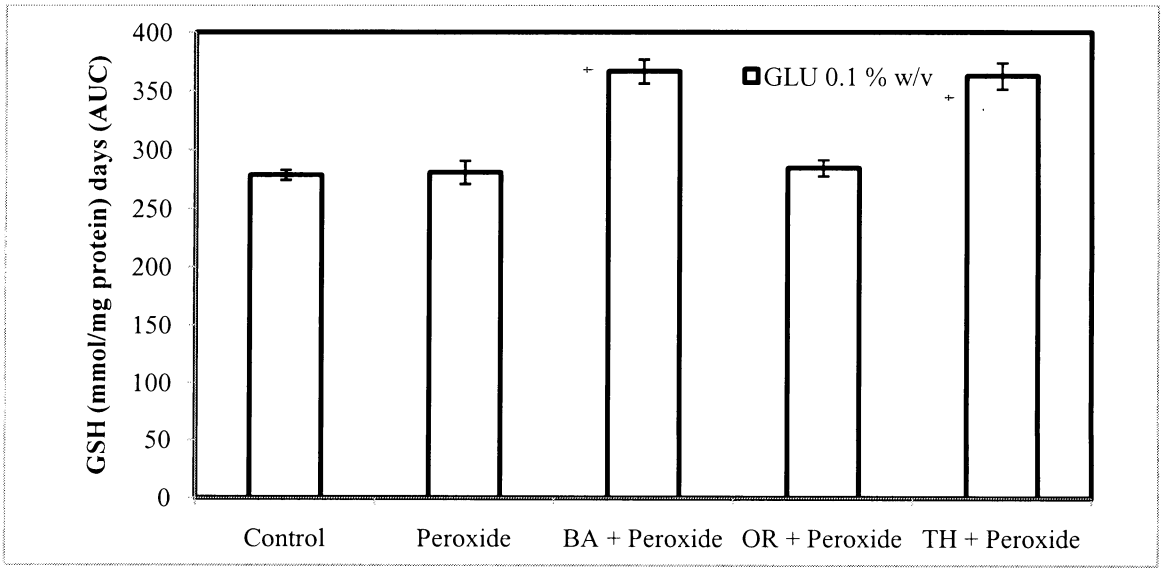

Figure 13: Overall Effectiveness of Different Lamiaceae Herbs on Glutathione Levels (GSH, GLU) Over 6 Days in Peroxide Stressed *L. terrestris* Over 6 Days
a- indicates significant difference between Treatment+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

Nitric Oxide

Nitric oxide plays a significant role in cell signaling and vasodilation in reproductive physiology. The effect of Lamiaceae herbs + 12mM peroxide on nitric oxide production in seminal vesicles was analyzed indirectly using a standard Griess test as previously described. Control worms had total levels of nitric oxide at 340.7 µmol/L/mg of protein and 296.8 µmol/L/mg of protein on day 2 and day 6 respectively (Table 16).

The treatment with 12mM hydrogen peroxide + LGM significantly lowered levels of nitric oxide when compared to controls. Nitric oxide levels were 204.9 µmol/L/mg of protein and 240. 5 µmol/L/mg of protein on day 2 and day 6 respectively, which were both significantly (p=0.0046; p=0.0011) lower than controls which only consumed LGM (Table 16). From AUC calculations, the 12mM peroxide treatment had significantly (p=0.0047) lower levels of nitric oxide than LGM treated control worms over the duration of 6 days (Figure 14).

Among all Lamiaceae herbs used, 0.1% (w/v) BA + PER was most effective at increasing nitric oxide levels when compared to 12mM peroxide. On day 2, the total nitric oxide values were 387.7 µmol/L/mg of protein, which was significantly (p=0.0034) higher than the 12mM peroxide treatment (Table 16). On day 6, the nitric oxide levels decreased to 247.8 µmol/L/mg of protein, which was significantly (p=0.0174) lower than the 12mM peroxide group (Table 16). It was shown that the basil + 12mM peroxide had significantly (p=0.0170) higher levels of nitric oxide than the 12mM peroxide treatment based on the AUC values (Figure 14). However when compared to control values, treatment with BA + PER significantly (p=0.0390; p=0.0011) lowered levels nitric oxide on day 2 and day 6, respectively (Table 16). The AUC indicated that over the course of 6

days, worms which consumed basıl + 12mM peroxide had significantly (p=0.0068) lower nitric oxide levels than controls (Figure 14).

Treatment with 0.1% (w/v) TH + PER also increased nitric oxide levels. Day 2 nitrıc oxide values were 331.9 µmol/L/mg of protein, which was significantly (p=0.0292) higher than the 12mM peroxide group (Table 16). On day 6, the nıtric oxide levels were 294.1 µmol/L/mg of protein. Upon observation of the AUC, it was noted that TH + PER had significantly (p=0.0190) higher levels of nitric oxide than 12mM peroxide (Figure 14). When compared to worms which consumed LGM, *L. terrestrıs* treated with TH + PER had significantly (p=0.0025; p=0.0077) lower levels of nıtric oxide on day 2 and day 6, respectively (Table 16). It was shown that treatment with TH + PER resulted in significantly (p=0.0064) lower nitric oxide values over the course of 6 days when compared to controls based on AUC values (Figure 14).

Worms which were treated with 0.1% (w/v) oregano + 12mM peroxide did differ in levels of nitric oxide when compared to 12mM peroxide. On the second day of treatment, nitric oxide values were 229.2 µmol/L/mg of protein, which was significantly (p=0.0402) lower than the 12mM peroxide group (Table 16). Nitric oxide levels on the sixth day of treatment were 317.7 µmol/L/mg of protein (Table 16). It was shown that there was no significant difference between OR + PER and 12mM peroxide over the duration of treatment as indicated by the AUC (Figure 14). Comparısons to controls revealed significantly (p=0.0074; p=0.0034) lower nitric oxide levels in OR + PER treated worms on day 2 and day 6, respectively (Table 16). Based on calculations of the AUC, over the 6 day treatment control worms had significantly (p=0.0046) higher levels of nitric oxide than OR + PER worms (Figure 14).

Table 16: Effect of Lamiaceae Herbs on Levels of Nitric Oxide (µmol/L/mg of protein) in Seminal Vesicles Tissues of Peroxide Stressed *Lumbricus terrestris*
* indicates Treatment + Peroxide is significantly lower than Peroxide/Control ; # indicates if Treatment + Peroxide is significantly higher than Peroxide/Control ($p< 0.05$)
†∗ indicates if Peroxide is significantly lower than Control; †#indicates if Peroxide is significantly higher than Control; n=7

Concentration % (w/v)		Nitric Oxide (µmol/L/mg of protein) Day 2	*P* value (T+P vs C)	*P* value (T+P vs P)	Day 6	*P* value (T+P vs C)	*P* value (T+P vs P)
Basil + Peroxide	0.1	387.7	0.0390*	0.0034#	247.8	0.0011*	0.0174*
Oregano+ Peroxide	0.1	229.2	0.0074*	0.0402*	317.7	0.0034*	0.1490
Thyme + Peroxide	0.1	331.9	0.0025*	0.0292#	294.1	0.0077*	0.1551
Peroxide	12mM H202 + LGM	204.9	0.0046†*		240.5	0.0011†*	
Control	LGM	340.7		-	296.8		-

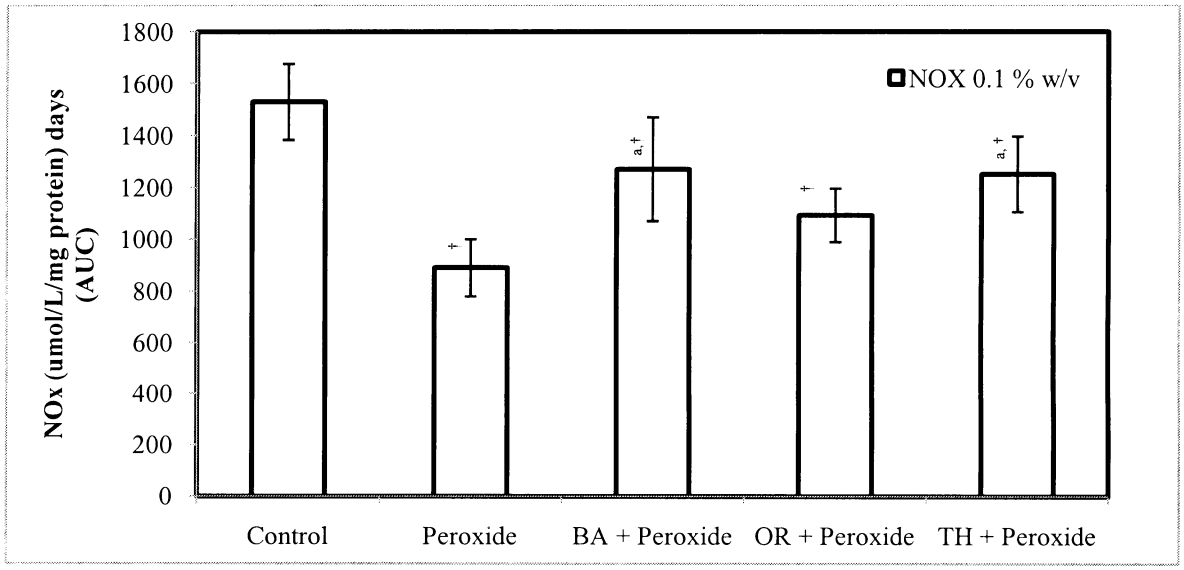

Figure 14: Overall Effectiveness of Different Lamiaceae Herbs on Nitric Oxide Levels (NOX) Over 6 Days in Peroxide Stressed *L. terrestris* Over 6 Days
a- indicates significant difference between Treatment+Peroxide and Peroxide over 6 days (AUC) ($p<0.05$)
†- indicates significant difference from the control (AUC) ($p<0.05$)
The data is represented as means ± SEM

Discussion

In Chapter I, five different Lamiaceae herbs were screened to determine their effects on redox and NO signaling in seminal vesicles of *Lumbricus terrestris*. It was reported that there were significant increases in nitric oxide production and antioxidant enzyme activity, and decreases in lipid peroxidation and sperm deformations after consumption of the dietary herbs. Given these results, the next objective was to determine if these herbs would have cytoprotective and genoprotective effects in a hydrogen peroxide induced oxidative stress. Recently, our lab has shown that the addition of 12mM hydrogen peroxide to standard LGM was sufficient to induce oxidative damages within the muscle tissues of *Lumbricus terrestris* (Hutton et al., 2009 and Thesis).

Therefore, in this study a similar concentration of hydrogen peroxide was added to the LGM and the same parameters were measured as previously described in Chapter I. Upon evaluation of the total effectiveness of treatment, it was observed that lipid oxidation increased as indicated by a 188.85% spike in MDA levels relative to controls (Table 17). In conjunction with increases in lipid oxidation, it was observed that there was a 30.47% increase in sperm deformations and an 8.58% increase in DNA fragmentation in worms consuming LGM with hydrogen peroxide (Table 17). Additionally, there was an 18.25% increase in SOD activity, a slight 0.76% increase in glutathione levels, and a 10.47% decrease in catalase activity in the seminal vesicles of worms feeding on LGM with peroxide. These results are consistent with that of a recent study in which the spermatozoa of infertile males was analyzed for lipid peroxidation levels and antioxidant enzyme activity. The researchers found that increases in lipid peroxidation were positively correlated with sperm deformations, and asthenozoospermic

samples also contained higher SOD and glutathione peroxidase activity (Dandekar et al., 2002). Similar to the results in this study, they showed catalase activity to be relatively unchanged. Dandekar et al. attributed the increased SOD and glutathione peroxidase activity as an attempt to overcome oxidative stress. In a similar fashion, the antioxidant response observed in this study may be a reaction to the acute oxidative stress induced by treatment with hydrogen peroxide. Additionally it is known that hydrogen peroxide is able to cause proteolysis of the NF-κB inhibitor protein, IKB, thereby enabling NF-κB to translocate to the nucleus and initiate transcription of target genes such as those for SOD (Das et al., 1995). This may be another mechanism by which SOD activity was increased after treatment with hydrogen peroxide. Also, nitric oxide levels were also significantly decreased by 41.78% in the peroxide treatment group. These results are in contrast to several studies that indicate the hydrogen peroxide induces expression and activity of eNOS and iNOS which increased nitric oxide levels (Michel, 2010; Arab et al., 2010).

The addition of Lamiaceae herbs to LGM containing hydrogen peroxide appears to ameloriate the effects of the acute oxidative stress induced in *L.terrestris*. Overall, in comparison to controls it was observed that there was an 8.92% increase in MDA levels, a 3.98% increase in DNA fragmentation, and a 5.56% decrease in sperm deformations (Table 17). This data suggests that there are slight increases in oxidative damages even with herb treatments; however, the extent of damage in comparison to the peroxide group is drastically reduced. When comparisons are made between the peroxide treatment and the herb + peroxide treatment it was observed that there was a 62.29% decrease in MDA levels, 27.61% decrease in sperm deformations, and a 4.23% decrease in DNA fragmentation in worms treated with Lamiaceae herbs (Table 18). These results are

similar to a recent *in vitro* study that indicated extracts of sage, oregano, and rosemary were able to protect Caco-2 cells against hydrogen peroxide induced DNA damage (Aherne et al., 2007).

It appears that phytochemicals present in Lamieace herbs may protect against oxidative damages induced by hydrogen peroxide by restoring redox homeostasis within the organism. In this study, it was reported that there was a 21.22% increase in GSH levels, a 7.84% increase in SOD activity, and a 19.19% decrease in catalase activity in the seminal vesicles of worms treated with herbs + peroxide relative to controls (Table 17). Although the antioxidant enzyme activity and GSH levels were increased when compared to controls, the antioxidant enzyme response was not as exaggerated in the herbs + peroxide group as in the peroxide group. This was indicated by an overall 12.73% decrease in SOD activity and a 9.74% decrease in catalase activity when compared to the peroxide group (Table 18). Previous research has shown that extracts of sage, rosemary, basil, oregano, and thyme are effective scavengers of free radicals, able to quench singlet oxygen, and chelate metals capable of participating in the Fention reaction (Triantaphyllou et al., 2001; Dorman et al., 2002; Matkowski and Piotrowska, 2006). Therefore, the protective benefits of these herbs which were observed in this study could be due, in part, to their free-radical scavenging abilities and reducing power. Interestingly, it was also observed that there were significant increases in GSH levels in the herb + peroxide treatment group. In comparison to the peroxide treatment, there was a 20.35% increase in GSH levels. As was predicted previously, these herbs may activate the antioxidant reponse element (ARE) via the transcription factor, Nrf2, which could lead to the transcription of γ-glutamylcysteine synthetase and increase levels of GSH

(Myhrstad et al., 2002). Recently, Masuntani et al showed that the Lamiaceae herb, *Perilla frutescens*, was able to activate the ARE via Nrf2 and prevent hydrogen peroxide induced cytotoxicity *in vitro* (Masutani et al., 2009).

Moreover, it was indicated that nitric oxide levels increased in response to treatment with herbs by 35.34% when compared to peroxide treatments; however NO levels remained lower than controls by 21.21%. Therefore, this provides further evidence that Lamiaceae herbs can stimulate nitric oxide production even in a situation where NO production is being depressed. As previously discussed, nitric oxide plays a vital role in the tissues of the seminal vesicles where eNOS expression is proposed to play an important role in junction restructuring and differentiation of germ cells into sperm cells (Lee and Cheng, 2004). This data suggests that phytochemicals from Lamiace herbs may stimulate nitric oxide signaling pathways, and contribute to several of the effects observed.

Table 17: The Effect of Different Lamiaceae Herbs + Hydrogen Peroxide on Antioxidant Enzyme Activity, Markers of Oxidative Stress and Nitric Oxide Biomarkers Relative to Controls.

SOD- Superoxide Dismutase, CAT- Catalase, GSH- Reduced Glutathione, MDA- Malondialdehye, DFO- Sperm Deformity, DFR- DNA Fragmentation, NOX- Nitric Oxide, BA- Basil, OR- Oregano, TH-Thyme

	Percent Change from Control Values (%)						
Treatment	**SOD**	**CAT**	**GSH**	**MDA**	**DFO**	**DFR**	**NOX**
Peroxide	18.25	-10.47	0 76	188.85	30 47	8 58	-41 78
BA + Peroxide	17.48	-20.10	31 46	25 04	8 24	3 51	-16 94
OR + Peroxide	3.55	-16.89	2 13	-12 57	-8.06	6 13	-28.51
TH + Peroxide	2.50	-20 58	30 08	14.29	-16.85	2.30	-18.18
AVG OF Herbs	**7.84**	**-19.19**	**21.22**	**8.92**	**-5.56**	**3.98**	**-21.21**

Table 18: The Effect of Different Lamiaceae Herbs + Hydrogen Peroxide on Antioxidant Enzyme Activity, Markers of Oxidative Stress and Nitric Oxide Biomarkers Relative to Peroxide Treatment.

SOD- Superoxide Dismutase, CAT- Catalase, GLU- Reduced Glutathione, MDA- Malondialdehye, DFO- Sperm Deformity, DFR- DNA Fragmentation, NOX- Nitric Oxide, BA- Basil, OR- Oregano, TH-Thyme

	Percent Change from Peroxide Values (%)						
Treatment	**SOD**	**CAT**	**GSH**	**MDA**	**DFO**	**DFR**	**NOX**
BA + Peroxide	-0 94	-10.75	30 46	-56.71	-17.03	-4 67	42 68
OR + Peroxide	-17.98	-7.18	1 36	-69.73	-29.53	-2 25	22.80
TH + Peroxide	-19 26	-11 29	29.10	-60 43	-36.26	-5.78	40 54
AVG OF Herbs	**-12.73**	**-9.74**	**20.31**	**-62.29**	**-27.61**	**-4.23**	**35.34**

CHAPTER III

THE PHYSIOLOGICAL EFFECTS OF COMBINATIONS OF LAMIACEAE HERBS ON REDOX AND NITRIC OXIDE SIGNALING IN SEMINAL VESICLES OF *LUMBRICUS TERRESTRIS*

Effects of Combinations of Phytochemicals

There has been a recent shift towards using plant-derived compounds or herbal medicines as a means of disease prevention and treatment as opposed to conventional Western medicine. The dissatisfaction with current Western medicine is due to several factors such as unpleasant side effects, lack of treatment success, and the rising cost of prescription drugs. Additionally, the ease of access of herbal medicines and the belief among the general population that these treatments are "natural" and safe contribute to their popularity and use (Stickel et al., 2005). Traditional Chinese medicine and several herbal supplements use processed, multi-component natural products in various combinations aimed at multiple targets of a disease (Lee, 2000). However, research regarding the interactions between bioactive compounds in natural products is limited, and the studies that have been conducted have demonstrated both antagonism and synergism among herb combinations (Chung et al., 2004; Adams et al., 2006). These properties associated with mixtures of various herbs may aid in the treatment of a disease or potentially cause unwanted adverse effects.

The additive or synergistic effects of natural products is due in part to the variety of chemical compounds which have the potential to act on several physiological processes associated with a disease (Kaufman et al., 1999; Briskin, 2000). Several *in vitro* studies with cancer cells lines have indicated combinations of secondary metabolites to be more effective anti-cancer agents than the single purified active compounds in the combination. Seeram et al. found that pomegranate juice had the greatest antioxidant and antiproliferative activity against human oral, colon, and prostate cancer cell lines than the major bioactive compounds in pomegranate juice, punicalagin and ellagic acid (Seeram et al., 2005). Additionally, one particular study in a mouse model of androgen-sensitive human prostate cancer found that a combination soy phytochemical concentrate and green tea synergistically inhibited tumor proliferation, angiogenesis, and metastasis (Zhou et al., 2003).

The synergistic and additive effects of herbal combinations open the door for future drug discoveries and treatments. However, there still remains a lack of scientific data on herbal and dietary supplements toxicity levels, proper dosage, and quality control. This is especially challenging for health care providers and poison control centers that treat and manage the adverse effects of these supplements (Haller, 2006). Also, many of the studies that have been conducted on herb combination treatments have been *in vitro*. These studies fail to take into account metabolic catabolism or interactions that increase bioavailability of these compounds *in vivo* (Chung et al., 2004).

Objective of Study

The objective of this study was to determine the effects of different combinations of Lamiaceae herbs on redox and NO signaling *in vivo* in seminal vesicles of *Lumbricus*

terrestris. Specifically, the effect of Lamiaceae phytochemicals on redox status, antioxidant enzymes (superoxide dismutase, catalase), oxidative damage to DNA, and NO mediated spermatogenesis.

Materials and Methods

Preparation of Media

Materials and methods were described previously in Chapter 1 (page 14). However, treatment plates contained two Lamiaceae herbs each at 0.5% (w/v) for a total concentration of 0.1% (w/v). Based on results from Chapter I, it was determined that treatment with Lamiaceae herbs at a concentration of 0.1% (w/v) was most effective in terms of modulating redox homeostasis. Also, combinations were comprised of the top three Lamiaceae herbs (basil (BA), oregano (OR), thyme (TH)) from Chapter I. The treatment groups were as follows: basil + oregano, oregano + thyme, and basil + thyme (Table 19).

Table 19: Lamiaceae Herb Combination Treatment for *Lumbricus terrestris*

COMBINATIONS	Herb % (w/v)	Herb %(w/v)	Total Concentration % (w/v)
Basil + Thyme (BA + TH)	BA (0 05)	TH (0.05)	0 1
Oregano + Thyme (OR + TH)	OR (0 05)	TH (0 05)	0.1
Oregano + Basil (OR + BA)	OR (0.05)	BA (0.05)	0.1

Results

Sperm Deformations

There are relatively few studies evaluating the effects of herbal combinations, even though multi-ingredient herbal supplements and formulations are commonly used (Tan and Vanitha, 2004). Based on previous evaluation of sperm deformations after a 6 day treatment with individual Lamiaceae herbs, significant changes in the ratio of deformed to normal cells occurred. Treament with combinations of two Lamiaceae herbs (basil + thyme, thyme + oregano, basil + oregano) at concentrations equal to 0.1% w/v also significantly altered the ratio of deformed spermatozoa to normal spermatozoa. *Lumbricus terrestris* only consuming lumbricus growth medium (LGM) for 6 days were established as controls in this study. After feeding for two days ad libitum, 22.8% of spermatozoa exhibited a deformation. On day 6, 23.75% of all spermatozoa evaluated had some type of deformation in control worms (Table 20)

The basil + thyme treatment over the span of 6 days resulted in the lowest percentage of sperm with abnormal morphologies in comparison to other treatments. On day 2 and 6 deformations were 22.5% and 23.7% respectively (Table 20). Upon observation of the overall effectiveness of this treatment, it was revealed that treatment with herb combination was not significantly different than the control based on calculations of the AUC (Figure 15).

L. terrestris treated with the mix of oregano + basil combination had increased levels of sperm morphological deformations over the duration of the 6 day treatment. On day 2 and day 6, deformities were calculated to be 27.2% and 28.2% respectively (Table

20). The control samples had significantly (p=0.0144) lower levels of deformations over the span of 6 days than the herb combination experimental group (Figure 15).

Treatment with oregano + thyme resulted in the highest number of sperm deformations. On day 2, 34.5% of all spermatozoa displayed morphological changes which were significantly (p= 0.0237) higher than controls on this day. Deformations on day 6 were 24.7% (Table 20). It was observed that over the duration of treatment, the control worms had significantly (p= 0.0104) lower percentages of sperm deformations than the treatment group (Figure 15).

Table 20: Effect of Lamiaceae Herb Combinations on Sperm Deformations
* indicates significantly lower than control; #significantly higher than control ($p< 0.05$); n=7

Concentration % (w/v)		Sperm Deformations (%)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	22.5	0.3104	23.7	0.1572
Oregano+ Thyme	0.1	34.5	0.0237#	24.7	0.4481
Oregano + Basil	0.1	27.2	0.0901	28.2	0.3138
Control	LGM	22.8	-	23.75	-

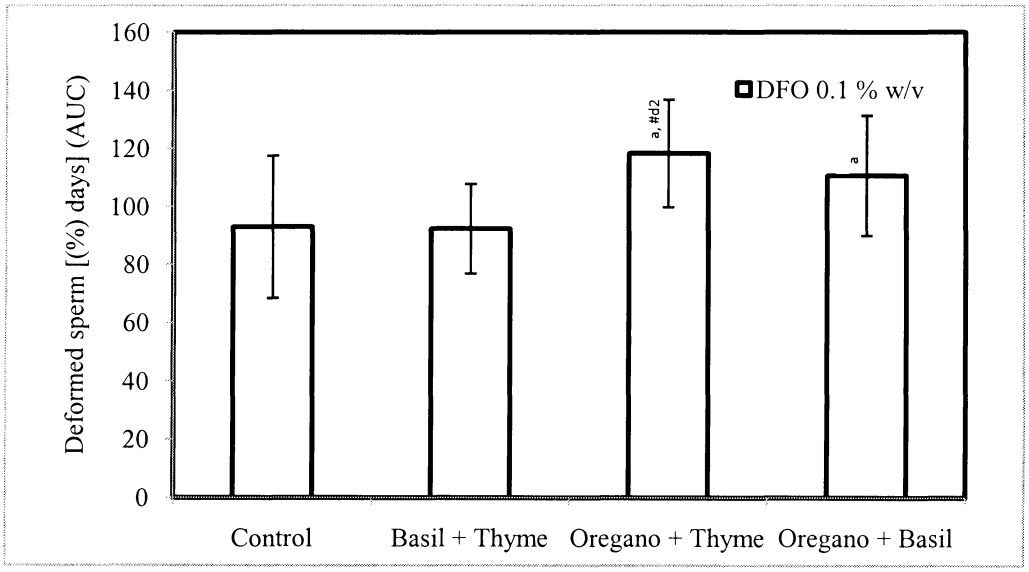

Figure 15: Overall Effectiveness of Lamiaceae Herb Combinations on Sperm Deformations (DFO) Over the Duration of 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Malondialdehyde Assay

The extent of lipid peroxidation in seminal vesicles was measured by TBARS to assess the amount of malondialdehyde formed. In control worms that fed on LGM the levels of MDA after 2 days was 0.6 mmol/mg of protein. On the 6^{th} day, the level of MDA decreased to 0.4 mmol/mg of protein (Table 21). Treatment with combinations of Lamiaceae herbs significantly increased levels of MDA.

Treatment with oregano + thyme resulted in less of an increase in lipid oxidation than the other herb combinations. On the second day of treatment MDA levels were 0.7 mmol/mg of protein, which was significantly (p=0.0001) higher than controls (Table 21). During the sixth day of testing, the lipid oxidation values for this treatment was 0.5 mmol/mg of protein which was also significantly (p=0.0003) higher when compared to controls (Table 21). Over the duration of the 6 day treatment, the controls had significantly (p=0.0021) lower levels of MDA than the treatment group (Figure 16).

The worms which fed on the basil + thyme media also had increases in MDA formation. Lipid oxidation levels were 1.0 mmol/mg of protein and 0.5 mmol/mg of protein on day 2 and day 6, respectively, which were significantly (p=0.0002; p=0.0018) higher when compared to controls (Table 21). It was observed that the controls had significantly (p=0.0030) less MDA formation than worms which consumed the herbal combination throughout the six days. (Figure 16).

Treatment with basil + oregano resulted in highest increase in the amount of lipid oxidation among all combinations. The level of MDA on day 2 was 1.1 mmol/mg of protein, which was significantly (p=0.0154) higher than controls (Table 21). On day 6, the level of MDA was also 1.1 mmol/mg of protein, which was increased significantly

(p=0.0002) in comparison to controls (Table 21). Over the course of 6 days, *L.terrestris* which only fed on LGM had significantly (p=0.0017) less lipid oxidation when compared to the herb treated group (Figure 16).

Table 21: Effect of Combinations of Lamiaceae Herbs on MDA (mmol/mg of protein) Formation in Seminal Vesicles as Measured by TBARS.
* indicates significantly lower than control; #significantly higher than control ($p< 0.05$); n=7

Concentration % (w/v)		MDA (mmol/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	1.0	0.0002#	0.5	0.0018#
Oregano+ Thyme	0.1	0.7	0.0001#	0.5	0.0003#
Oregano + Basil	0.1	1.1	0.0154#	1.1	0.0002#
Control	LGM	0.6	-	0.4	-

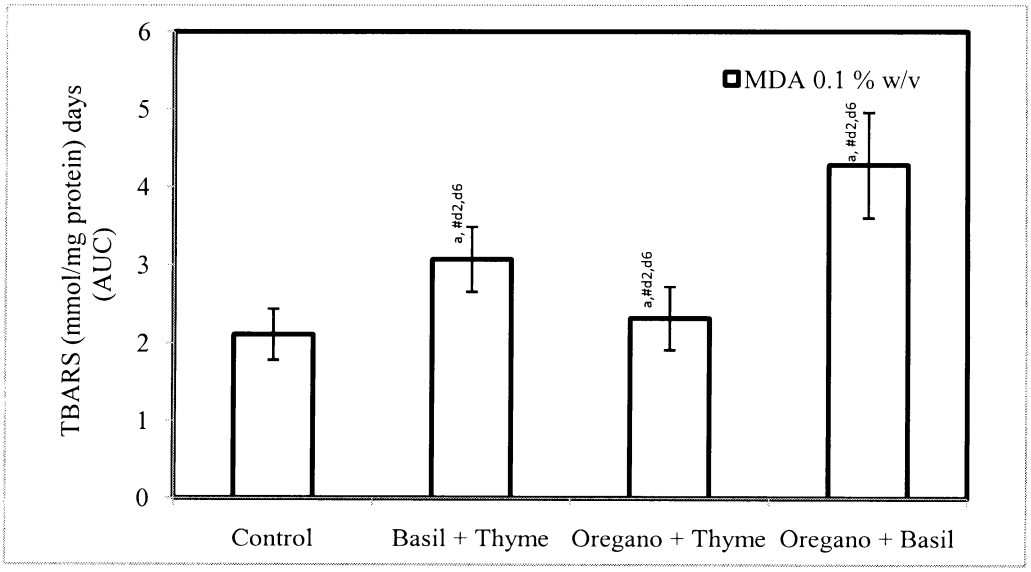

Figure 16: Overall Effectiveness of Combinations of Lamiaceae Herbs on Levels of MDA in Seminal Vesicles Over 6 Days Measured as TBARS
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

DNA Fragmentation

In order to assess the extent of free radical induced damage to spermatozoa, the percentage of DNA fragmentation was measured. In control worms that fed on LGM , it was shown that on the second day of treatment the amount of DNA fragmentation was 46.0 %. On day 6, the extent of fragmentation decreased to 44.9%. In *L. terrestris* treated with combinations of Lamiceae herbs, the percentage of DNA fragmentation was altered significantly.

Treatment with the combination of oregano + thyme lowered the percentage of DNA fragmentation over the duration of 6 days. On day 2 and day 6 the amount of fragmented DNA was 45.2% and 38.3% respectively (Table 22). It was observed that the amount of fragmentation in the treatment group was significantly (p=0.0297) lower than controls over the span of 6 days (Figure 17).

When *L. terrestris* fed on the oregano + basil combination, the day 2 and day 6 treatments had opposite effects in terms of DNA fragmentation. On day 2 the level of fragmented DNA was 48.8% which was significantly (p=0.0134) increased when compared to day 2 controls. The sixth day of treatment resulted in fragmentations of 41.9% which was significantly (p=0.0126) decreased in comparison to the controls. Overall, it has been observed that the amount of DNA fragmentation in the herb combination group was not significantly different than controls as determined by the AUC (Figure 17).

Among all of the combinations, basil + thyme significantly increased DNA fragmentation over the duration of treatment. On day 2 and day 6 the amount of fragmentation was 49.3% and 50.4% respectively, which were both significantly

(p=0.0001; p=0.0001) higher than controls (Table 22). It was concluded that the controls had significantly (p=0.0268) lower levels of DNA fragmentation than the experimental group over the 6 day treatment (Figure 17).

Table 22: Effect of Combinations of Lamiaceae Herb Treatments on DNA Fragmentation (%)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		DNA Fragmentation (%)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	49.3	0.0001#	50.4	0.0001#
Oregano+ Thyme	0.1	45.2	0.2035	38.3	0.0508
Oregano + Basil	0.1	48.8	0.0134#	41.9	0.0126*
Control	LGM	46.0	-	44.9	-

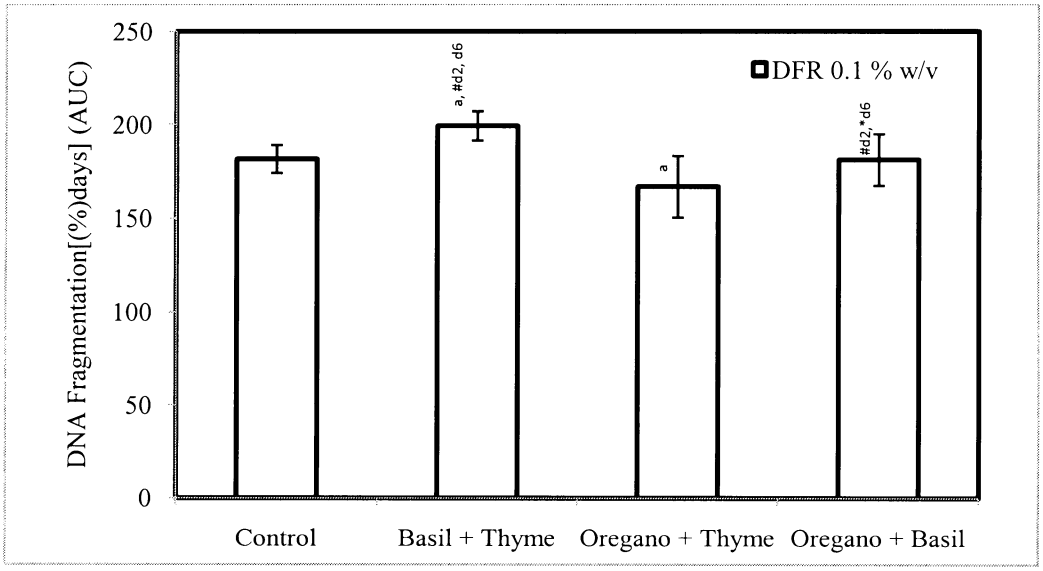

Figure 17: Overall Effectiveness of Combinations of Lamiaceae Herbs on Levels of DNA Fragmentation (DFR) Over 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Superoxide Dismutase

The superoxide dismutase (SOD) activity in seminal vesicles was quantified using the NBT/diformazan assay as discussed previously. In control worms on day 2, the level of diformazan formed was 111.4 µmol/mg of protein. Tests on day 6 revealed that the levels of diformazan had decreased to 89.7 µmol/mg of protein.

The combination of oregano + thyme was effective at reducing the formation of diformazan. On day 2, the level of diformazan was 88.4 µmol/mg of protein, which was significantly (p=0.0096) lower than controls (Table 23). On the sixth day, diformazan levels were 75.2 µmol/mg of protein, which was also significantly (p=0.0143) decreased in comparison to controls (Table 23). Upon determination of the AUC, it was observed that the herb combination treatment had significantly (p=0.0030) lower levels of diformazan than the control group (Figure 18).

The basil + thyme combination was also effective at increasing SOD activity as indicated by the lower levels of diformazan formation. Treatment with the herb combination resulted in levels of diformazan at 87.2 µmol/mg of protein and 83.0 µmol/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0032; p=0.0081) lower than controls (Table 23). The herb combination treatment had significantly (p=0.0121) lower diformazan levels in comparison to controls over the duration of treatment as indicated by AUC values (Figure 18).

L. terrestris treated with the combination of oregano + basil also had decreased levels of diformazan over the 6 day treatment. On the second day, diformazan levels were 95.3 µmol/mg of protein, which was significantly (p=0.0117) lower than controls (Table 23). On day 6, the amount of diformazan formed was 93.8 µmol/mg of protein (Table

23). Overall, it was observed that the herb combination treatment had significantly (p=0.0408) lower levels of diformazan than controls over the duration of 6 days (Figure 18).

Table 23: Effect of Combinations of Lamiaceae Herbs on Superoxide Dismutase Activity as Indicated by Levels of Diformazan (µmol/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Diformazan (µmol/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	87.2	0.0032*	83.0	0.0081*
Oregano+ Thyme	0.1	88.4	0.0096*	75.2	0.0143*
Oregano + Basil	0.1	95.3	0.0117*	93.8	0.1243
Control	LGM	111.4	-	89.7	-

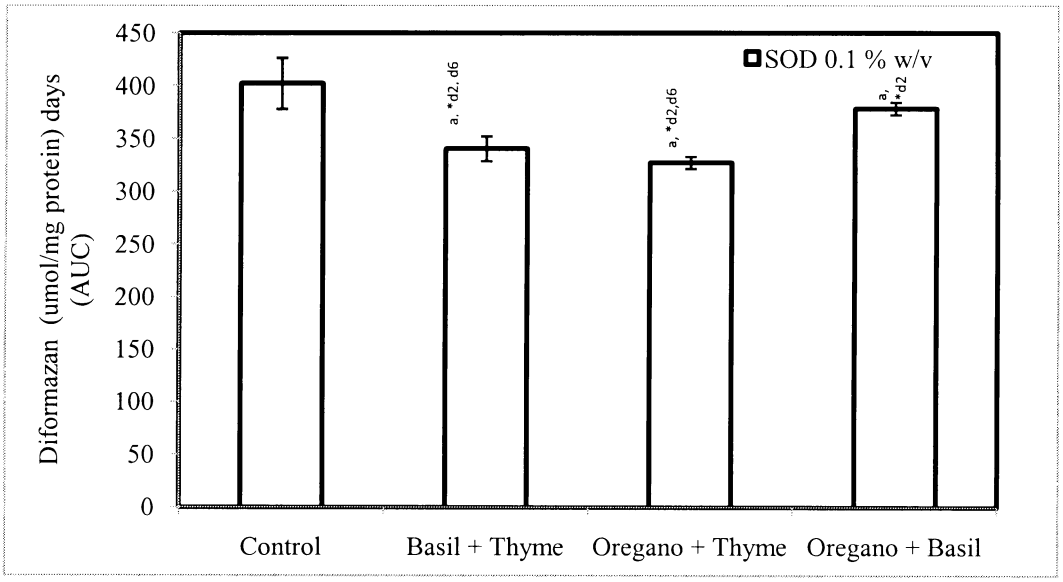

Figure 18: Overall Effectiveness of Lamiaceae Herb Combination Treatment on Superoxide Dismutase Activity as Indicated by Levels of Diformazan Over 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Catalase

Removal of hydrogen peroxide, a powerful reactive oxygen species and product of SOD mediated reactions, requires the enzymatic action of catalase. In control worms which fed only on LGM, the catalase activity was 295.4 mU/ mg of protein after two days. The catalase activity after six days of treatment was 278.6 mU/mg of protein. Treatment with herb combinations resulted in lowered catalase activity on all days.

The decrease in catalase activity was less in basil + thyme than the other combinations. On day 2 and day 6 the catalase activity was 271.2 mU/mg of protein and 271.0 mU/mg of protein, which was significantly (p=0.0006; p=0.0009) lower than controls (Table 24). Upon calculation of the AUC, it was observed that over the duration of treatment, the controls had significantly (p=0.0312) higher catalase activity than the basil + thyme combination (Figure 19).

The oregano + thyme combination also decreased catalase activity over the 6 day span. The catalase activity was 288.1 mU/mg of protein and 246.4 mU/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0000; p=0.0000) lower in comparison to the controls (Table 24). Overall, the catalase activity of the controls was significantly (p=0.0257) higher than the combination treatment based on calculation of the AUC (Figure 19).

Lumbricus terrestris treated with the oregano + basil combination had the lowest catalase activity over the duration of treatment. On day 2, catalase activity was 248.4 mU/mg of protein which was significantly (p=0.0000) decreased in comparison to day 2 controls (Table 24). On the sixth day of treatment, the catalase activity was 263.6 mU/mg of protein, which was also significantly (p=0.0003) lowered when compared to the

controls (Table 24). As was the case with the other herb combinations, the overall effect of the oregano + basil combination was a significant (p=0.0201) decrease in catalase activity when compared to the control (Figure 19).

Table 24: Effect of Combinations of Lamiaceae Herbs on Catalase Activity (mU/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Catalase (mU/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	271.2	0.0006*	271.0	0.0009*
Oregano+ Thyme	0.1	288.1	0.0000*	246.4	0.0000*
Oregano + Basil	0.1	248.4	0.0000*	263.6	0.0003*
Control	LGM	295.4	-	278.6	-

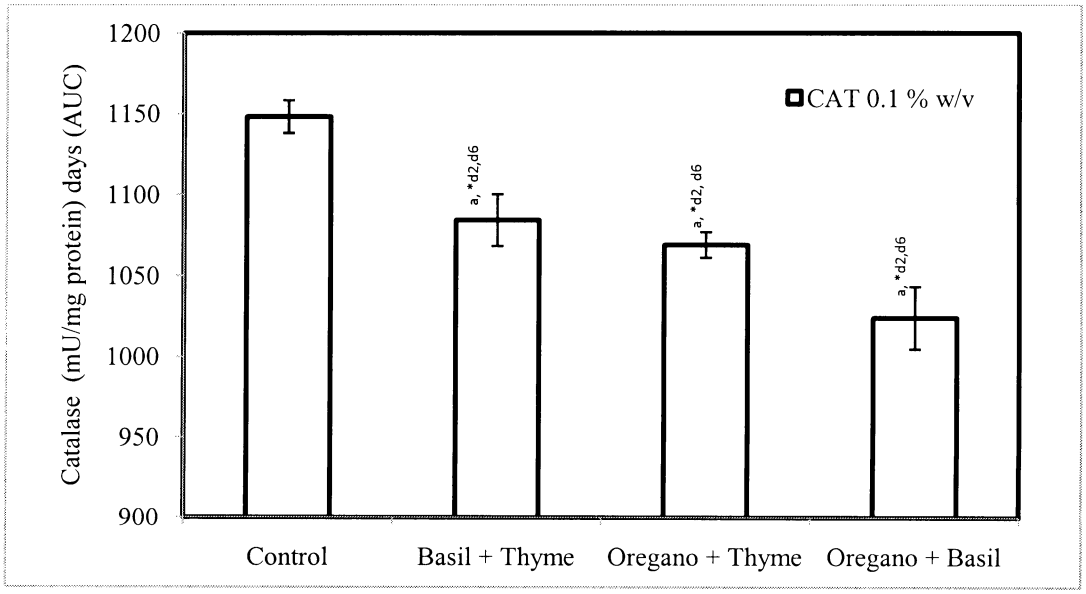

Figure 19: Overall Effectiveness of Lamiaceae Herbs Combinations on Catalase Activity Over 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Glutathione

Glutathione plays an important role in many biological processes and is a critical cellular antioxidant involved in maintaining cytosolic redox homeostasis. In control worms, on day 2 the level of glutathione was 72.2 mmol/mg of protein. On day 6 the amount of glutathione decreased to 67.2 mmol/mg of protein. Overall, the herb combination treatments were able to significantly increase glutathione levels when compared to controls.

Treatment with oregano + basil combination resulted in the most significant increases of glutathione in comparison to the controls. Levels of glutathione were 72.2 mmol/mg of protein and 88.4 mmol/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0001) higher than controls on day 6 (Table 25). From AUC values it was noted that treatment with this herb combination significantly (p=0.0115) increased glutathione levels in comparison to controls (Figure 20).

L. terrestris which fed on the basil + thyme combination also had increased levels of glutathione. On day 2 glutathione levels were 79.8 mmol/mg of protein (Table 25). On the sixth day of treatment, the level of glutathione was 78.3 mmol/mg of protein which was significantly (p=0.0005) increased when compared the day 6 controls (Table 25). It was observed that the herb combination group had significantly (p=0.0199) higher levels of glutathione than controls based on the AUC (Figure 20).

The combination of oregano + thyme also caused a significant change in glutathione levels over the duration of treatment. On the second day, glutathione levels were 66.1 mmol/mg of protein which was significantly (0.0018) lowered when compared to day 2 controls (Table 25). However on the sixth day of treatment, the levels of

glutathione increased to 82.4 mmol/mg of protein which was significantly (p=0.0030) increased in comparison to controls (Table 25). The overall effectiveness of treatment with herb combination resulted in significantly (p=0.0394) increased levels of glutathione when compared to the controls (Figure 20).

Table 25: Effect of Lamiaceae Herb Combinations on Levels of Glutathione in the Tissues of Seminal Vesicles
* indicates significantly lower than control; #significantly higher than control($p<0.05$); n=7

Concentration % (w/v)		Glutathione (mmol/mg of protein) Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	79.8	0.0932	78.3	0.0005#
Oregano+ Thyme	0.1	66.1	0.0018*	82.4	0.0030#
Oregano + Basil	0.1	72.2	0.0767	88.4	0.0001#
Control	LGM	72.2	-	67.2	-

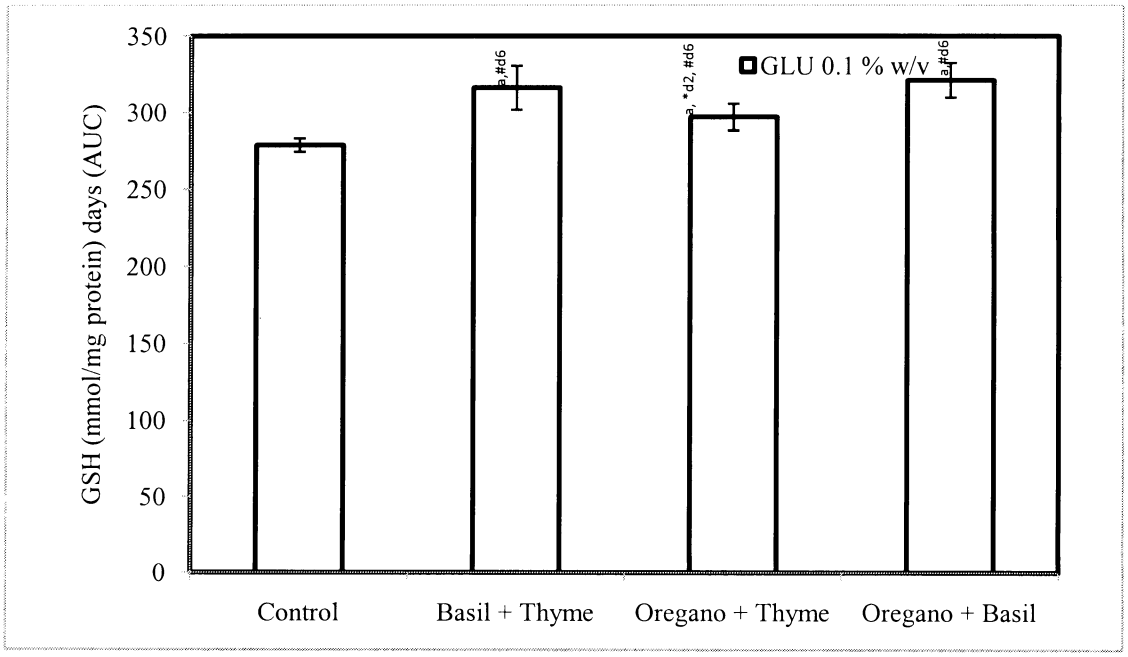

Figure 20: Overall Effectiveness of Different Lamiaceae Herb Combinations on Glutathione Levels (GSH, GLU) Over 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means $\pm$ SEM

Nitric Oxide

Nitric oxide plays a significant role in cell signaling and vasodilation in reproductive physiology. The effect of Lamiaceae herb combinations on nitric oxide production in seminal vesicles was analyzed indirectly by determining total nitrate/nitrite levels using a standard Griess test. Control worms had total levels of nitric oxide at 340.7 µmol/L/mg of protein and 296.8 µmol/L/mg of protein on day 2 and day 6 respectively.

The treatment with a combination of basil + thyme significantly lowered levels of nitric oxide when compared to controls. Nitric oxide levels were 204.6 µmol/L/mg of protein and 185.8 µmol/L/mg of protein on day 2 and day 6, respectively, which was significantly (p=0.0003; p=0.0010) lower than controls (Table 26). From AUC calculations, the herb combination treatment had significantly (p=0.0038) lower levels of nitric oxide than LGM treated control worms (Figure 21).

Worms that consumed the oregano + basil combination also had decreases in levels of nitric oxide. With this herb combination, the total nitric oxide values were 230.6 µmol/L/mg of protein and 161.1 µmol/L/mg of protein on day 2 and day 6 respectively, which was significantly (p=0.0016) lower than controls on the second day (Table 26). It was shown that the control samples had significantly (p=0.0040) higher levels of nitric oxide than the oregano + basil combinations based on the AUC values (Figure 21).

The oregano + thyme combination resulted in the greatest decreases in nitric oxide levels. On day 2 the nitric oxide values were 147.5 µmol/L/mg of protein, which was significantly (p=0.0078) lower than the controls (Table 26). On day 6, the level of nitric oxide was 190.6 µmol/L/mg of protein and it was also significantly (p=0.0025)

decreased in comparison to the control (Table 26). This herb combination significantly (p=0.0033) lowered nitric oxide levels when compared to the controls as noted from AUC values (Figure 21).

Table 26: Effect of Lamiaceae Herb Combinations on Levels of Nitric Oxide (µmol/L/mg of protein)
* indicates significantly lower than control; #significantly higher than control ($p<0.05$); n=7

Concentration % (w/v)		Nitric Oxide (µmol/L/mg of protein)			
		Day 2	*P* value	Day 6	*P* value
Basil + Thyme	0.1	204.6	0.0003*	185.8	0.0010*
Oregano+ Thyme	0.1	147.5	0.0078*	190.6	0.0025*
Oregano + Basil	0.1	230.6	0.0502	161.1	0.0016*
Control	LGM	340.7	-	296.8	-

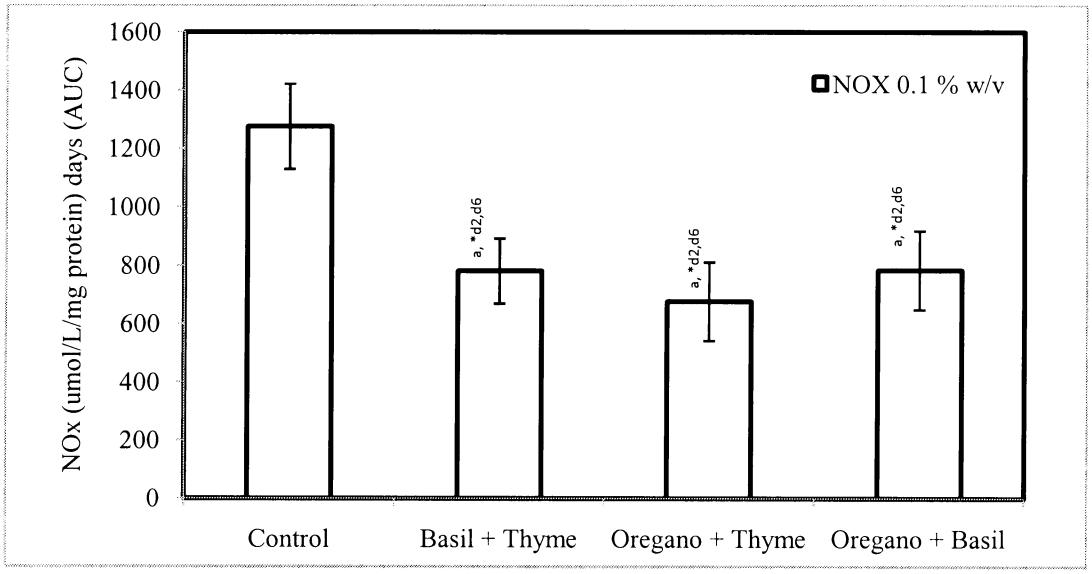

Figure 21: Overall Effectiveness of Lamiaceae Herb Combinations on Total Nitric Oxide Levels Over 6 Days
a- indicates significant difference between control and treatment over 6 days (AUC) ($p<0.05$)
*- indicates significantly lower than the control ($p<0.05$)
#- indicates significantly higher than the control ($p<0.05$)
d2= day 2; d6= day6 – indicates days when treatment was significantly different from control ($p<0.05$)
The data is represented as means ± SEM

Discussion

In traditional eastern medicine, formulations contained combinations of several natural ingredients aimed at treating various illnesses (Stickel et al., 2005; Adams et al., 2006). The philosophy behind combination therapies is that these formulations have increased potentency due to synergistic interactions occurring between compounds within each individual ingredient (Lee, 2000; Chung et al., 2004). Consistent with these ideas, recent *in vitro* studies have shown that combinations of bioactive compounds can in fact display synergism (Mertens-Talcott et al., 2003; Zhou et al., 2003; Seeram et al. 2005). Additionally, emerging research suggests that phytochemicals present in a whole food matrix or mixture of whole foods confer more health benefits than individual, purified phytochemicals (Hennekens et al., 1996; Liu, 2003; Vattem et al., 2006). It is speculated that phytochemicals in combinations mutually enhance their functionalities (Vattem et al., 2005). In support of this, Vattem et al. have shown that a blend of cranberry juice extract with grape seed, blueberry, and oregano extracts was far more superior at inhibiting *H. pylori* activity than purified phenolic compounds at similar doses (Vattem et al., 2004). However upon evaluation of the literature, it is obvious that there remains a lack research examining the effects of herbal combinations *in vivo*. In Chapter I it was reported that significant changes in redox and nitric oxide signaling occured in response to treatment with individual Lamiaceae herbs *in vivo*. In the current study, combinations of the most effective herbs from Chapter I were evaluated for their effects on antioxidant enzyme activity, markers of oxidative stress, and nitric oxide biomarkers in *Lumbricus terrestris*.

The overall effect of Lamiaceae herb combinations was assessed and reported as a percent change from control values (Table 27). In contrast to treatment with individual herbs, it was observed that were significant increases in markers of oxidative stress after combination treatment. In comparison to controls, MDA levels increased by 52.75%, sperm deformations increased by 52.75%, and DNA fragmentation increased by 0.49% (Table 27). The noted increases in markers of oxidative stress were coupled with 13.33% increase in SOD activity, 11.70% increase in GSH levels, and a 7.75% decrease in catalase activity. The increase in SOD activity and GSH may be a response to oxidative stress induced by the herbal combinations. Consistent with a previous study, GPx and SOD activity were increased in the spermatozoa of infertile men with signs of oxidative stress (Dandekar et al., 2002). The data suggests that treatment with herb combinations may have caused a synergistic interaction among phytochemicals. Previous studies have indicated that interactions among phytochemicals can lead to increases in bioavailability. It has been shown in rat and human studies that the bioavailability of curcumin, a phytochemical found in turmeric, can be increased by 154-2000% upon co-administration with piperine, an alkaloid found in pepper (Shoba et al., 1998; Anand et al., 2007). Additionally, piperine has also been shown to inhibit glucuronidation of epigallocatechin gallate (EGCG) and slow down gastrointestinal transit, thereby increasing absorption and bioavailability (Lambert et al., 2004). Furthermore, it is also known that furanocoumarins found in citrus fruits such as grapefruits are capable of inhibiting the drug metabolizing enzyme, CYP3A4, thereby increasing bioavailability of certain drugs (Nowak, 2008). In the current study, the net dosage of Lamiaceae phytochemicals was the same in herb combination treatments as in individual herb treatments, however the net effect was

completely opposite. The observed physiological effects in herb combination treatments may be a direct result of increased *in vivo* concentrations of phytochemicals. These compounds are treated as foreign molecules (xenobiotics) and rapidly metabolized in the liver by drug-metabolizing phase I enzymes such as cytochrome P450 dependent oxidases (Stickel et al., 2005). It is known that these drug metabolizing enzymes are capable of producing highly reactive metabolites which can interact with proteins and DNA and induce oxidative stress (Loeper et al., 1993; Stickel and Seitz, 2000). It has also been shown that agents found in natural products are capable of inducing cytochrome P450 enzymes, and subsequently causing cytoxic effects (Stedman, 2002; Stickel et al., 2005). A recent study with purified ellagic acid revealed that treatment of Caco-2 cells at high concentrations induced expression and activity of the phase I enzyme, CYP1A1 (Gonzlez-Sarras et al., 2009). Additionally, the *in situ* exposure of ellagic acid also induced cytochrome p450 activities in the colon of rats. These results suggest that ellagic acid could potentially be procarcinogenic at higher concentrations (Vattem and Shetty, 2005). It is possible, given the results in this study that similar effects are occurring as a result of synergistic interactions among Lamiaceae phytochemicals. Additionally, we observed a significant decrease in nitric oxide biomarkers in herb combination treatment groups by 41.43% (Table 27).

In summary, combinations of Lamiaceae herbs appeared to induce oxidative stress in seminal vesicle tissues of *Lumbricus terrestris*. In future studies lower concentrations of Lamiaceae herbs will be utilized to see if reductions in oxidative stress can be achieved. Additionally, an isobologram will help identify if there are potential synergistic interactions occurring among Lamiaceae phytochemicals.

Table 27: The Effect of Combinations of Different Lamiaceae Herbs on Antioxidant Enzyme Activity, Markers of Oxidative Stress and Nitric Oxide Biomarkers Relative to Controls.

SOD- Superoxide Dismutase, CAT- Catalase, GLU- Reduced Glutathione, MDA- Malondialdehye, DFO- Sperm Deformity, DFR- DNA Fragmentation, NOX- Nitric Oxide, BA- Basil, TH- Thyme, OR- Oregano

	Percent Change from Control Values (%)						
Treatment	**SOD**	**CAT**	**GSH**	**MDA**	**DFO**	**DFR**	**NOX**
BA + TH	15.38	-5.55	13 38	45.54	-0.72	9.77	-38 76
OR + TH	18 68	-6.88	6 55	9.88	27.24	-8 09	-46.96
OR + BA	5.94	-10.81	15.16	102.83	19.00	-0.22	-38.56
AVG OF Herbs	**13.33**	**-7.75**	**11.70**	**52.75**	**15.17**	**0.49**	**-41.43**

CHAPTER IV

EFFECT OF DIFFERENT LAMIACEAE HERBS ON REDOX SIGNALING PATHWAYS IN *CAENORHABDITIS ELEGANS*

Invertebrate Model for Natural Product Research: *Caenorhabditis elegans*

Natural products, especially Lamiaceae herbs, have exhibited several bioactive properties which have been elucidated through many *in vitro* studies. Although these studies have contributed greatly to what is known about Lamiaceae phytochemicals, they fail to take into account the complex metabolic processes that occur *in vivo* (Lampe and Chang, 2007). The majority of *in vivo* data on natural products has been conducted in rodent models. Although a reliable model, these studies tend to be costly and require proper facilities and ethical requirements for their use. Recently the invertebrate, *C. elegans*, has emerged as a highly efficient alternative for natural product research (Wilson et al., 2006; Kampkotter et al., 2007; Pietsch et al., 2009). In 1998 the genome of *C. elegans* was completely sequenced and data revealed a substantial conservation of biological mechanisms between other animal species (Hope, 1999). Therefore, identification of a molecular mode of action by which plant secondary metabolites possess the proposed neuroprotective, anti-inflammatory, chemopreventative, and other beneficial properties can potentially be elucidated in this model. The ease of use of *C. elegans* makes it an appealing model system. These nematodes have minimal growth requirements therefore maintenance is inexpensive; they can be flexibly manipulated for

experimental procedures and do not require stringent ethical requirements for their use (Wilson et al., 2010). Currently, several transgenic strains of *C. elegans* exist which have Green Fluorescent Protein (GFP) in conjunction with promoters for various proteins (Link et al., 1999). Therefore, changes in gene expression in response to various treatments can be visualized and quantified.

Through previous *in vivo* work with *Lumbricus terrestris* it was speculated that Lamiaceae phytochemicals altered redox and nitric oxide signaling via an insulin-like signaling pathway and an ARE mediated pathway. In order to further understand the mechanism of redox modulation by Lamiaceae herbs several transgenic strains of *C. elegans* with GFP promoter constructs of genes relevant to these pathways were used (Table 28).

Table 28: Different Transgenic Strains of *C. elegans* Carrying Promoter GFP Fusions for Relevant Genes in Redox Pathways

Strain	Genotype	Protein	Role of Gene
GR1352	daf-16(mgDf47)	DAF-16α::GFP	Encodes transcription factor interacting with IGF-1 genes. Regulates CAT, SOD.
TJ356	zIs356IV	DAF-16::GFP	Regulates expression of SOD,CAT (increase) AOX decrease gene
CF1553	muIs84	SOD-3::GFP	mitochondrial Fe/Mn SOD; dismutation of superoxide
GA800	wuIs151	CTL-1 + CTL-2 + CTL-3 + MYO-2::GFP	produces 10 times more catalase; negatively regulated by insulin signaling
AA278	dhIs59	DAF-9::GFP	Cytochrome P450
CL2120	dvIs14	MTL-2::GFP	Metallothionein-2. Involved in metal detox & stress. Regulates fertility
CL2122	dvIs15	MTL-2::GFP	mtl-2 but no amyloid peptides

Table 28: Continued

CL2070	dvls70	HSP-16.2::GFP	Heat shock protein expressed in response to heat for protection/interacts w/β-amyloid peptides.
BC14279	dpy-5(e907) I;sEx14279	ISP-1::GFP	Iron-sulfur protein. Member of ETC (cytochrome) in complex III. Low levels: lower sensitivity to ROS.
BC13348	dpy-5(e907)I; sEx13348	GST-4::GFP (driven by GST)	Glutathione-S-transferase. AOX enzyme, req. for sperm motility.
VC337	gcs-1(ok436)/mIn1[mIs14 dpy-10(e128)] II	GCS-1::GFP	gamma glutamine cysteine synthetase; rate limiting enzyme in glutathione synthesis
OH7631	nhr-67(ok631) IV; otEx3362	ELT-2::GFP	nuclear hormone receptor that regulates linker cell migration; determines the shape of the male gonad and sperm release
DZ325	ezIs2 III; him-8(e1489) IV	FKH-6::GFP	transcription factor that promotes male gonadal cell fates in XO animals. Loss of function of the fkh-6 results in feminized males.

These particular strains of *C. elegans* contain GFP constructs for genes downstream of the *daf-2* insulin-like signaling pathway. The *daf-2* gene codes for an insulin-like/IGF-1 receptor which has been conserved from *C. elegans* to mammals, along with all components of this signaling pathway (Lee et al., 2001; Murphy, 2006; Gami and Wolkow, 2006). Activation of insulin-like pathway begins with the binding of peptide hormones, insulin or insulin like growth factor (IGF-1), to insulin receptors (*daf-2*) that activate phosphoinositide 3-kinase (PI3K)(*age-1*) (Samson and Wong, 2002; Gami and Walkow, 2006). P13K then generates phosphoinositide-3,4,5-P_3 and

phosphoinositide-3,4-P_2 (PIPs) which activate serine/threonine kinases, AKT-1,-2, phosphoinositide-dependent kinase (PDK-1), and serum and glucocorticoid-induced protein kinase (SGK-1) (Gami and Walkow, 2006). SGK-1 is activated via phosphorylation by PDK-1 and then binds to AKT to form a multi-protein complex that is capable of phosphorylating downstream proteins (Figure 22) (Hertweck et al., 2004). This pathway has been extensively studied and is important in the regulation of reproduction, metabolism, and stress resistance (Henderson and Johnson, 2001; Murphy et al., 2003; Gami et al., 2006). Studies on aging have indicated that a decrease in insulin signaling, which occurs in DAF-2 mutations, significantly increases the life span of *C.elegans* (Kenyon et al., 1993). Through investigations into the mechanisms behind increases in longevity, it has been shown that insulin signaling prevents the nuclear translocation of DAF-16, a forkhead box transcription factor (FOXO) homolog found in *C. elegans* (Lee et al., 2001; Libina et al., 2003; Gami et al., 2006). Microarray analysis and comparative genomics have revealed several genes for antioxidant and detoxification enzymes regulated by *daf-16* and they include catalases (*ctl-1, ctl-2*), superoxide dismutases (*sod-1, sod-3*), metallothioneins (*mtl-1, mtl-2*), and heat shock proteins (*hsp-16*) (Munoz, 2003; Murphy et al., 2003; Hertweck and Baumeister, 2005). This provides some evidence that an increased resistance to oxidative stress may contribute to longevity. Additionally, this resistance to oxidative stress can possibly be enhanced by bioactive compounds in natural products. It has also been shown that *C. elegans* treated with *Acanthopanax senticosus* and *Rhodiola rosea,* had increased stress resistance and lifespan by inducing translocation of *daf-16* (Weigant et al., 2009).

In addition to an active insulin-like pathway in *Lumbricus terrestrisis,* it was speculated that activation of the antioxidant response element occured via the transcription factor Nrf2. The phase II detoxification response is a conserved mechanism consisting of several enzymes which scavenge free radicals utilizing glutathione (Tullet et al., 2008). In a state of oxidative stress, Nrf proteins accumulate in the nucleus of a cell to induce expression of phase II enzymes (An and Blackwell, 2003; Inoue et al., 2005). Interestingly bioactive compounds from natural products, such as sulphoraphane, have also been shown to induce Nrf2 dependent gene expression in cell cultures (Wagner et al., 2010). Researchers have identified an Nrf2 orthologous protein in *C. elegans*, SKN-1, which also induces transcription of phase II detoxification enzymes (An et al., 2005; Tullet et al., 2008). It has been shown that this transcription factor accumulates in the nucleus following a p38 mitogen-activated protein kinase (MAPK) signaling cascade. In this process, SKN-1 is phosphorylated by PMK-1 and able to translocate to the nucleus, thereby inducing expression of target genes (Inoue et al., 2005). The genes under regulation of SKN-1 include gamma glutamyl-cysteine synthetase (*gcs-1)*, iron sulphur protein (*isp-1)*, NADH quinine oxidoreductase, glutathione S-transferase (*gst-4*), superoxide dismutase (*sod-1)*, and catalase (*ctl-1*) (An and Blackwell, 2003; Kell et al., 2007). However in the absence of oxidative stress, SKN-1 is negatively regulated by glycogen synthase kinase-3 (GSK-3)(An et al., 2005). Moreover, inhibition of SKN-1 has also been shown occur via an insulin/IGF-1-like signaling mechanism. As is the case in DAF-16 inhibition, the multi-protein complex, AKT-1,-2 and SGK-1, phosphorylate SKN-1 causing the protein to be retained within the cytosol (Figure 22) (Tullet et al., 2008).

In Chapter I, it was observed that were significant increases in antioxidant ezyme activity and nitric oxide metabolites following a 6 day treatment with different Lamiaceae herbs in the seminal vesicle tissues of *Lumbricus terrestris*. These physiological changes occurred alongside significant decreases in biomarkers of oxidative damage. The bulk of experimental data with Lamiaceae herbs fails to identify a possible mechanism of action by which these herbs are able to modulate physiological processes. Through work with *L. terrestris*, it was proposed that Lamiaceae phytochemicals altered redox and nitric oxide signaling via an insulin-like signaling pathway and an ARE mediated pathway. As previously stated, *C. elegans* are an established model by which molecular modes of action can be easily identified. Given the genetic homology and ease of use of this organism, it was possible to utilize this model to monitor changes in expression of proteins relevant to the insulin-like signaling pathway following treatment with different Lamiaceae herbs.

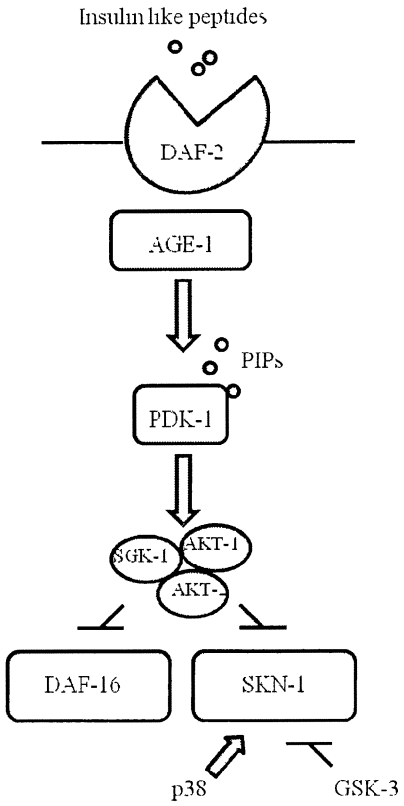

Figure 22: Inhibition of DAF-16 and SKN-1 by insulin-like signaling in *C elegans.* (Tullet et al., 2008)

Objective of Study

The objective of this study was to understand the mechanism of redox modulation by Lamiaceae herbs using transgenic strains of *Caenorhabditis elegans* with GFP promotor constructs of relevant genes.

Materials and Methods

Nematode Propagation and Treatment

Transgenic strains of *Caenorhabditis elegans* with GFP promoter constructs were obtained from the Caenorhabditis Genetics Center. All strains were propagated on 35mm or 60mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl,

0.25% Peptone, 1M CaCl, 1M $MgSO_4$, 5mg/mL Cholesterol in ethanol, 1M KPO_4) at 18-20°C (Brenner, 1974). The NGM media was poured aseptically into culture plates using a peristaltic pump and allowed to solidify for 36 hours. NGM culture plates were then inoculated with 50 µl of *Escherichia coli* OP50 overnight cultures and incubated for 9 hours at 37°C. The strains of *C. elegans* were maintained by picking 2-3 adult worms onto freshly inoculated NGM plates every 4-7 days.

Treatment plates contained water extractions of Lamiaceae herbs at concentrations of 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v). Water extractions of Lamiaceae herbs were prepared by heating 1.5g of the herb in 30mL of distilled water at 60°C for 30 minutes. The extract was then filtered through sterile 0.2µm filters to avoid contamination. Extracts of Lamiaceae herbs were added to the NGM solution just prior to pouring (Caldicott et al., 1994). The treatment plates were inoculated with *E. coli* OP50 as previously described, and the cultures were also supplemented with herb extracts at the same concentration as the NGM. Two mature adult worms were then transferred to treatment plates (3 plates of each concentration) and allowed to lay eggs, hatch and grow to the L4 to mature adult stage.

Fluorescence Imaging and Quantification

Images of two adult worms from each plate were captured using the Nikon SMZ1500 fluorescence microscope with Ri1 CCD camera. Prior to capturing the images, the worms were temporarily immobilized by chilling the cultures on ice for 5 minutes. The relative fluorescence with respect to control was then quantified using the National Institute of Health's ImageJ software (Wolkow, 2007).

Statistical Analysis

Fold change in relative fluorescence was calculated between controls and each treatment concentration. Statistical analysis of data was performed using a two tailed Student's *t* test. p values ≤ 0.05 were considered statistically significant.

Results

Effect of Lamiaceae herbs on expression of DAF-16 in the strain TJ356

TJ356 is a transgenic *C. elegans* strain with integrated green fluorescent protein (GFP) fused to the last amino acid of the DAF-16a2 protein (*daf-16*::GFP) (Henderson and Johnson, 2001). As previously mentioned, DAF-16 is a transcription factor that is analogous to mammalian FOXO proteins and plays a critical role in the oxidative stress response, reproduction, and aging in *C. elegans* (Murphy et al., 2003; Tullet et al., 2008). It has been shown that insulin and insulin-like growth factor signaling negatively regulate the nuclear translocation of DAF-16 through AKT/PKB, thereby inhibiting the functions of this transcription factor (Libina et al, 2003; Hertweck and Baumeister, 2005).

In this study, the expression of *daf-16* after treatment with different Lamiaceae herbs at concentrations of 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) was measured. Treatment with basil significantly altered expression *daf-16* in *C. elegans*. At 0.1% (v/v) of basil, DAF-16 had a 1.6 fold change that was significantly (p=0.0028) higher than controls (Table 29). Basil at 0.5% (v/v) resulted in a 1.3 fold change which was also significantly (p=0.0372) higher than worms on NGM (Table 29). Worms treated with 1.0% (v/v) basil had a 0.7 fold change, which was significantly (p=0.0379) lower than control worms on NGM (Table 29).

It was shown that worms propagated on NGM supplemented with oregano had no significant change in *daf-16* expression. At 0.1% (v/v) there was a 1.1 fold change (p=0.3073), treatment at 0.5% (v/v) had 1.0 fold (p=0.9090), and 1.0% (v/v) of oregano had a 1.0 fold change (p=0.8861) in comparison to control (Table 29).

In response to treatment with rosemary, *daf-16* expression, for the most part, did not change significantly in comparison to controls. Treatment at 0.1% (v/v) of rosemary had a 0.9 fold change which was not significantly (p=0.0663) different than the control (Table 28). Expression of *daf-16* at 0.5% (v/v) of rosemary was 1.1 fold higher, but was not significantly (p=0.1833) different from controls (Table 29). However rosemary at 1.0% (v/v) resulted in a 1.0 fold change *daf-16* expression, which was significantly (p=0.0119) lower than worms on NGM (Table 29).

NGM supplemented with sage or thyme did not result in significant changes in *daf-16* expression. It was indicated that worms feeding on sage at 0.1% (v/v) and 1.0% (v/v) had a 0.9 fold (p=0.4496) and 0.5 fold (p=0.0541), respectively (Table 29). However at 0.5% (v/v) of sage, there was a 0.7 fold change which was significantly (p=0.0334) lower than the control (Table 29). *daf-16* expression was also significantly (p=0.0435) lower in worms which fed on 0.1% (v/v) thyme when compared to controls (Table 29). *C. elegans* grown on 0.5% (v/v) thyme had a 1.0 fold change (p=0.8314) and at 1.0% (v/v) had a 0.8 fold change (p=0.1066) (Table 29).

Table 29: Effect of Different Lamiaceae Herbs on *daf-16* Expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

Concentration % (v/v)		TJ356 (daf-16::GFP)	
		FC-RF	*P* value
Basil	0.1	1.6	0.0028*
	0.5	1.3	0.0372*
	1.0	0.7	0.0379†
Oregano	0.1	1.1	0.3073
	0.5	1.0	0.9090
	1.0	1.0	0.8861
Rosemary	0.1	0.9	0.0663
	0.5	1.1	0.1833
	1.0	1.0	0.0119†
Sage	0.1	0.9	0.4496
	0.5	0.7	0.0334†
	1.0	0.5	0.0541
Thyme	0.1	0.8	0.0435†
	0.5	1.0	0.8314
	1.0	0.8	0.1066

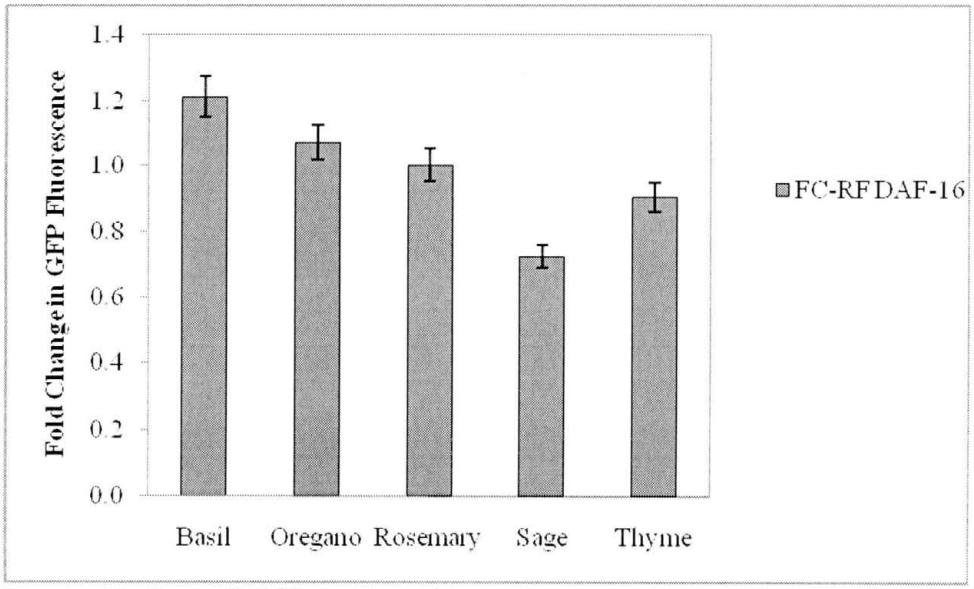

Figure 23: Overall Effect of Lamiaceae Herbs on DAF-16 Expression in *C. elegans*. Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of DAF-16α in the strain GR1352

The strain GR1352 has a GFP fusion to one of the isoforms of *daf-16*, *daf-16α*. Expression of this isoform occurs in almost all somatic cells, as opposed to the ß-isoform which is primarily expressed in the pharynx (Lee et al., 2001).

The data revealed that treatment with basil at all concentrations significantly increased expression of *daf-16α*. Expression was up-regulated 1.3 fold (p=0.0000), 1.2 fold (p=0.0015), and 1.1 fold (p=0.0098) in worms feeding 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) basil, respectively (Table 30).

In *C. elegans* feeding on oregano and rosemary there was no significant change in *daf-16α* expression with any of the concentrations used. Oregano at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in a 1.0 fold (p=0.8301), 0.9 fold (p=0.3288), and 1.0 fold change (p=0.7975), respectively (Table 30). Treatment with rosemary at 0.1% (v/v) and 0.5% (v/v) resulted in a fold change of 0.9 (Table 30). Worms feeding on rosemary at 1.0% (v/v) had a slight increase in *daf-16α* expression with 1.0 fold change, however it was not significantly (p=0.6690) different from controls (Table 30).

Worms which fed on NGM supplemented with sage had significantly increased *daf-16α* expression when compared to controls. Sage at 0.1% (v/v) (p=0.0047), 0.5% (v/v) (p=0.0001), and 1.0% (v/v) (p=0.0000) resulted in a 1.3 fold increase in *daf-16α* in comparison to worms on NGM only (Table 30).

For *C. elegans* propagated on culture plates supplemented with thyme, decreases in *daf-16α* expression were observed. At the lowest concentration, 0.1% (v/v), there was a 0.8 fold change, however it was not significantly (p=0.1384) different from controls

(Table 30). Treatment with thyme at 0.5% (v/v) and 1.0% (v/v) had a 0.6 fold (p=0.0130) and 0.7 fold change (p=0.0104), respectively (Table 30).

Table 30: Effect of Different Lamiaceae Herbs on *daf-16α* Expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

Concentration % (v/v)		GR1352 (DAF16α::GFP)	
		FC-RF	*P* value
Basil	0.1	1.3	0.0000*
	0.5	1.2	0.0015*
	1.0	1.1	0.0098*
Oregano	0.1	1.0	0.8301
	0.5	0.9	0.3288
	1.0	1.0	0.7975
Rosemary	0.1	0.9	0.1225
	0.5	0.9	0.0643
	1.0	1.0	0.6690
Sage	0.1	1.3	0.0047*
	0.5	1.3	0.0001*
	1.0	1.3	0.0000*
Thyme	0.1	0.8	0.1384
	0.5	0.6	0.0130†
	1.0	0.7	0.0104†

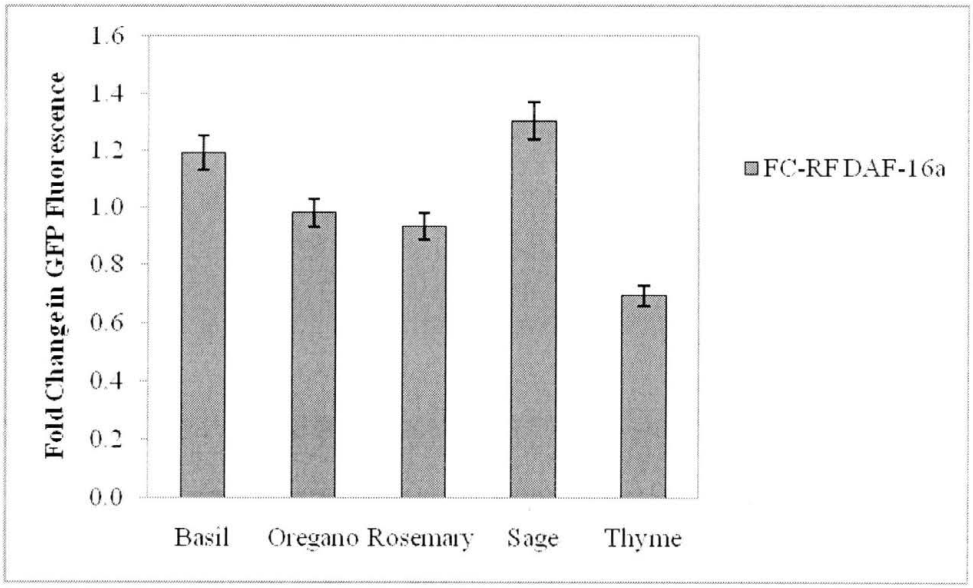

Figure 24: Overall Effect of Lamiaceae Herbs on DAF-16α Expression in *C. elegans*. Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Superoxide dismutase-3 (SOD-3) in the strain CF1553

The transgenic strain, CF1553, has a GFP integrated within *sod-3*, the gene which encodes a mitochondrial iron/manganese SOD (Giglio et al., 1994; Suzuki et al. 1996). It has been shown that DAF-16 can directly bind to the promoter region of *sod-3*, and initiate transcription of this important antioxidant enzyme (Libina et al., 2003; Doonan et al., 2008). Expression of *sod-3* was quantified after treatment with different Lamiaceae herbs.

For worms treated with basil, *sod-3* expression remained relatively unchanged (Table 30). At 0.1% (v/v) and 0.5% (v/v) of basil there was a 1.1 fold (p=0.3266) and 1.0 (p=0.9976) fold change, respectively (Table 30). However at the highest concentration 1.0% (v/v) there was a 0.7 fold change in relative fluorescence, which was significantly (p=0.0147) lower than controls (Table 31).

Treatment with oregano had variable effects on the expression of *sod-3*. At the lowest concentration of oregano, 0.1% (v/v), there was a 1.3 fold increase in *sod-3* expression which was significantly (p=0.0409) higher than worms on NGM (Table 31). Treatment with 0.5% (v/v) of oregano, resulted in a 0.9 fold change (p=0.3474). However with the concentration of oregano at 1.0% (v/v), there was a fold change of 0.8, which was significantly (p=0.0480) lower than controls (Table 31).

C. elegans treated with rosemary, for the most part, exhibited a decreased expression of *sod-3* (Table 31). In worms on culture plates supplemented with rosemary at 0.1% (v/v), *sod-3* expression was 0.8 fold different (p=0.1551) from controls (Table 31). With the concentration of rosemary increased to 0.5% (v/v) and 1.0% (v/v), *sod-3*

expression significantly decreased 1.0 fold (p=0.0181) and 0.9 fold (p=0.0043), respectively (Table 31).

After treatment with sage or thyme, there were no significant changes in *sod-3* expression observed when compared to controls. There was a 0.9 fold (p=0.1607), 0.9 fold (p=0.0686), and 1.0 fold (0.5241) in *sod-3* expression after treatment with sage at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v), respectively (Table 31). Treatment with thyme, similarly, resulted in no significant changes in *sod-3* expression. At 0.1% (v/v) of thyme, there was a 1.0 fold change (p=0.8355) observed (Table 31). At the higher concentrations 0.5% (v/v) and 1.0% (v/v), there was 1.1 fold (p=0.1891) and 0.9 fold (p=0.1425) change in *sod-3* expression, respectively (Table 31).

Table 31: Effect of Different Lamiaceae Herbs on *sod-3* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		CF1553 (sod-3::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	1.1	0.3266
	0.5	1.0	0.9976
Basil	1.0	0.7	0.0147†
	0.1	1.3	0.0409*
	0.5	0.9	0.3474
Oregano	1.0	0.8	0.0480†
	0.1	0.8	0.1551
	0.5	1.0	0.0181†
Rosemary	1.0	0.9	0.0043†
	0.1	0.9	0.1607
	0.5	0.9	0.0686
Sage	1.0	1.0	0.5241
	0.1	1.0	0.8355
	0.5	1.1	0.1891
Thyme	1.0	0.9	0.1425

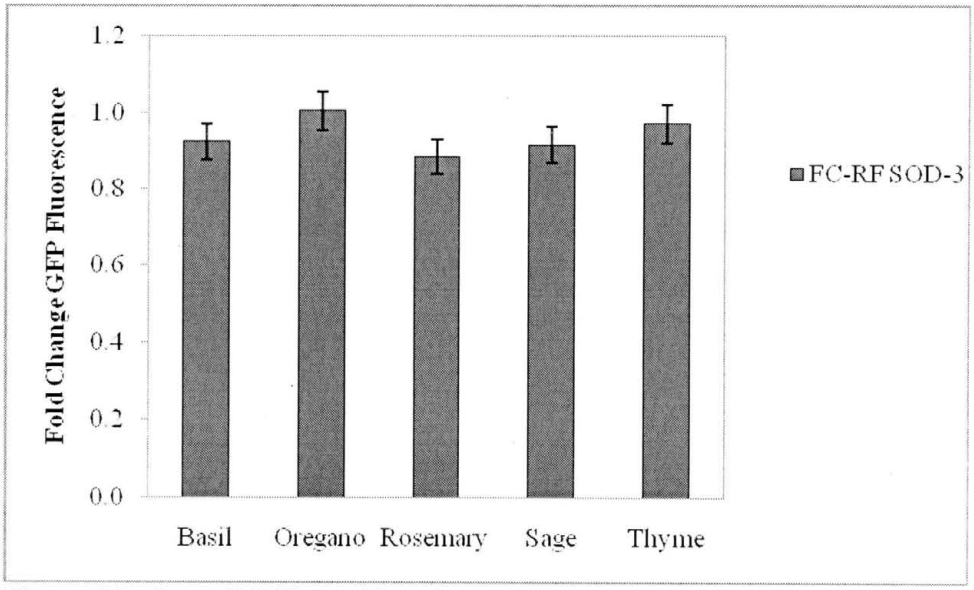

Figure 25: Overall Effect of Lamiaceae Herbs on SOD-3 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Catalase-1,-2,-3 (CAT-1,-2,-3) in the strain GA800

The expression of catalase genes, *ctl-1, ctl-2,* and *ctl-3* were measured using the transgenic strain, GA800. This strain was generated to produce a 10-fold increase in catalase activity in order measure the effect of H_2O_2 on aging (Doonan et al., 2008). It has been shown that insulin signaling negatively regulates expression of these genes through phosphorylation of the FOXO ortholog, DAF-16 (Doonan et al., 2008).

It was shown that worms treated with basil had slight increases in expression of the catalase genes. At 0.1% (v/v) there was a 1.4 fold increase in relative fluorescence, which was significantly (p=0.0001) higher when compared to controls (Table 32). Also at 0.5% (v/v) of basil, there was a significant (p=0.0140) increase in expression as indicated by a 1.3 fold change (Table 32). However at 1.0% (v/v) of basil, there was a 1.2 fold change which was not significantly (p=0.0816) different from worms on NGM (Table 32).

For worms feeding on oregano at 0.1% (v/v), there was a 1.0 fold change, but fluorescence was not significantly (p=0.9421) different from the control (Table 32). However at 0.5% (v/v), there was a 1.1 fold increase observed that was significantly (p=0.0390) different from worms on NGM (Table 32). Similarly, treatment with 1.0% (v/v) of oregano resulted in a 1.1 fold change, however it was not different (p=0.3175) from the control (Table 32).

Treatments with rosemary, sage, and thyme resulted in no significant changes in the expression of the catalase genes in comparison to the controls. Rosemary at the concentrations of 0.1% (v/v) and 0.5% (v/v) both had 0.9 fold changes, which was not significantly (p=0.1990; p=0.1318) different from controls (Table 32). However,

treatment with rosemary at 1.0% (v/v) resulted in a 0.9 fold change which was significantly (p=0.0390) lower than worms on NGM (Table 32). For worms feeding with sage supplemented plates at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v), catalase expression was lowered 1.0 fold (p=0.9946), 1.0 fold (p=0.5396), and 0.9 fold (p=0.3879), respectively (Table 32). *C. elegans* propagated on thyme plates, similarly, had lower catalase expression, however it was not significantly different from controls. Thyme at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in a 1.0 fold change for all concentrations (Table 32).

Table 32: Effect of Different Lamiaceae Herbs on *ctl-1, ctl-2, and ctl-3* Expression in *C. elegans*.

Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)

*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)

†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		GA800 (ctl-1 + ctl-2 + ctl-3 + myo-2::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	1.4	0.0001*
	0.5	1.3	0.0140*
Basil	1.0	1.2	0.0816
	0.1	1.0	0.9421
	0.5	1.1	0.0390*
Oregano	1.0	1.1	0.3175
	0.1	0.9	0.1990
	0.5	0.9	0.1318
Rosemary	1.0	0.9	0.0390†
	0.1	1.0	0.9946
	0.5	1.0	0.5398
Sage	1.0	0.9	0.3879
	0.1	1.0	0.0953
	0.5	1.0	0.4850
Thyme	1.0	1.0	0.9691

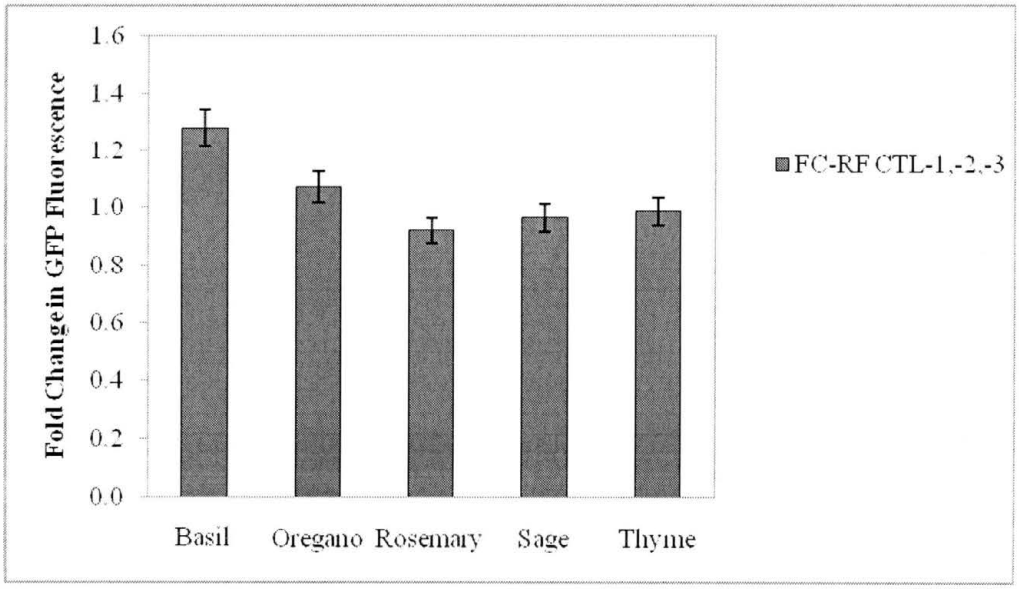

Figure 26: Overall Effect of Lamiaceae Herbs on CTL-1,-2,-3 Expression in *C. elegans*. Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of DAF-9 in the strain AA278

The *C. elegans* strain, AA278, has a GFP fusion integrated with *daf-9*. The *daf-9* gene codes for a cytochrome P450 hydroxylase, which has been shown to be involved in the detoxification of xenobiotic compound and the synthesis of steroid hormones (Jia et al., 2002). It is thought that DAF-9 generates a lipophilic hormone which binds to DAF-12 to promote reproductive development and regulation of dauer formation (Jia et al., 2002). DAF-9 is the point of convergence downstream of DAF-2/insulin/IGF receptor and DAF-7/TGFbeta, the two signaling pathways that control dauer formation (Albert and Riddle, 1988; Jia et al., 2002).

It was shown that *daf-9* expression was significantly increased upon treatment with basil at all concentrations. At 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) there was a 1.2 fold increase (p=0.0000), 1.2 fold increase (p=0.0000), and 1.1 fold increase (p=0.0000), respectively (Table 33).

For worms treated with oregano, *daf-9* expression did not change significantly from controls. Oregano at 0.1% (v/v), resulted in a 1.0 fold change (p=0.4267). At the increased concentration of 0.5% (v/v) and 1.0% (v/v), there was a 1.0 fold change in *daf-9* expression, however it was not significantly (p=0.3022;p=0.1486) different from controls (Table 33).

C. elegans cultured on plates supplemented with rosemary also had significantly increased expression of *daf-9*. Rosemary at 0.1% (v/v), resulted in a 1.0 fold increase in *daf-9*, which was significantly (p=0.0067) higher than worms on NGM (Table 33). After treatment with 0.5% (v/v) of rosemary, *daf-9* expression significantly (p=0.003)

increased 1.0 fold (Table 33). The expression of *daf-9* also increased 1.0 fold (p=0.0161) with the concentration of rosemary at 1.0% (v/v) (Table 33).

For worms propagated on sage plates, no significant changes in *daf-9* expression were observed. Treatment with sage at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v), resulted in a 0.9 fold change in *daf-9* expression, which was not significantly (p=0.2964; p=0.2248; p=0.2270) from controls (Table 33).

daf-9 expression was significantly higher than controls following treatment with with 0.1% (v/v) and 0.5% (v/v) of thyme by 1.1 fold (p=0.0132) and 1.1 fold (p=0.0149), respectively (Table 33). However at the highest concentration of thyme, 1.0% (v/v), there was a 1.0 fold change in *daf-9* expression, which was not different (p=0.0837) from controls (Table 33).

Table 33: Effect of Different Lamiaceae Herbs on *daf-9* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		AA278 (daf-9::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	1.2	0.0000*
	0.5	1.2	0.0000*
Basil	1.0	1.1	0.0000*
	0.1	1.0	0.4267
	0.5	1.0	0.3022
Oregano	1.0	1.0	0.1486
	0.1	1.0	0.0067*
	0.5	1.0	0.0003*
Rosemary	1.0	1.0	0.0161*
	0.1	0.9	0.2964
	0.5	0.9	0.2248
Sage	1.0	0.9	0.2270
	0.1	1.1	0.0132*
	0.5	1.1	0.0149*
Thyme	1.0	1.0	0.0839

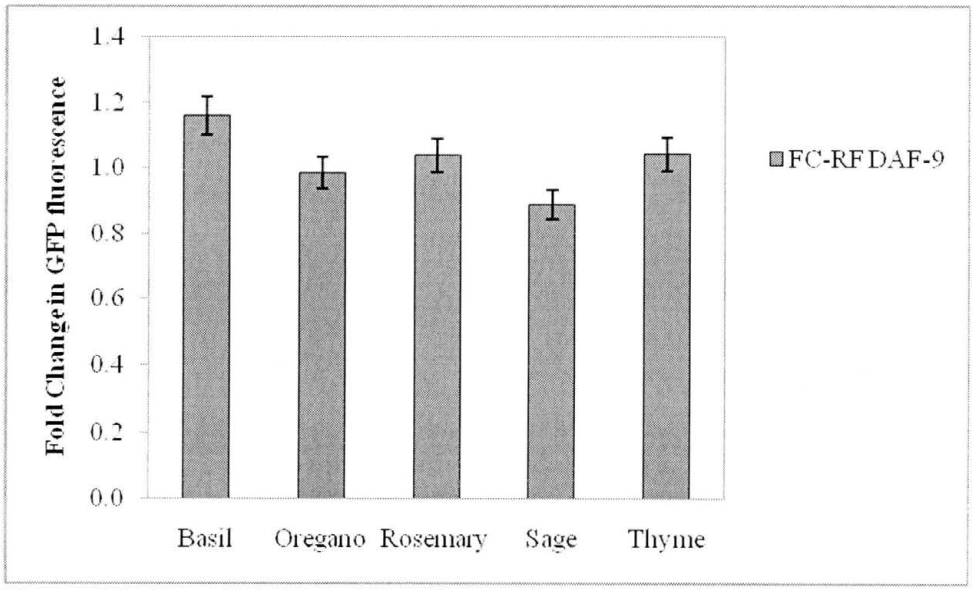

Figure 27: Overall Effect of Lamiaceae Herbs on DAF-9 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Metallothionein-2 (MTL-2) in the strain CL2120

CL2120 is a transgenic strain of *C. elegans* with a GFP promoter fusion bound to *mtl-2*. This particular strain also expresses human amyloid beta peptides (AB), and was generated to study the effects of metals on the aggregation of AB, a common cause of toxicity in Alzheimer's disease (McColl et al., 2009). Metallothioneins are important in protection against heavy metal toxicity and the subsequent ROS that may be generated from them (Kagi and Shaffer, 1988; Barsyte et al., 2001). These proteins are usually expressed in response to oxidative stress, metal ions, and inflammation (Hamer, 1986). In *C. elegans* there are two isoforms, MTL-1, which is constitutively expressed, and MTL-2, which is inducibly expressed when these animals are exposed to metals (Freedman et al., 1993; Barsyte et al., 2001). It has been shown in *daf-2* mutants that DAF-16 downregulates expression of *mtl-2* (Murphy et al., 2003).

Through evaluation of the data it was shown that treatment with basil significantly downregulated expression of *mtl-2* at all concentrations. Basil at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in a 0.8 fold decrease (p=0.007), a 0.8 fold decrease (p=0.0004), and a 0.6 fold decrease (p=0.0000), respectively (Table 34).

Similarly, *mtl-2* expression in *C. elegans* treated with oregano was lowered when compared to controls. At 0.1% (v/v) of oregano, there was a 1.0 fold change in *mtl-2* expression, however it was not significantly (p=0.8078) different from controls (Table 34). Oregano at concentrations of 0.5% (v/v) and 1.0% (v/v), resulted in 0.7 fold (p=0.0182) and 0.8 fold (p=0.0000) decrease in *mtl-2* expression.

For worms feeding on rosemary, *mtl-2* expression was also downregulated. Rosemary at 0.1% (v/v), resulted in a 0.9 fold change in *mtl-2* expression which was not

significantly (p=0.4358) different from worms on NGM (Table 34). However, 0.5% (v/v) and 1.0% (v/v) of rosemary significantly lowered *mtl-2* expression 0.8 fold (p=0.0127) and 0.7 (p=0.0274), respectively (Table 34).

In worms grown on sage treatment plates, *mtl-2* expression was significantly lowered by 0.4 fold (p=0.0200) at a concentration of 0.1% (v/v). However increasing concentrations of sage to 0.5% (v/v) and 1.0% (v/v) resulted in a 0.6 fold (p=0.0749) and 0.5 fold change (p=0.0812) in *mtl-2* expression, respectively, which was not significantly different come the controls (Table 34).

After treatment with thyme, *mtl-2* expression was relatively downregulated in comparison to controls. At 0.1% (v/v) of thyme, there was a 0.9 fold decrease in protein expression, which was significantly (p=0.0146) lower than worms on NGM (Table 34). Additionally, worms treated with thyme at a concentration of 0.5% (v/v) had a 0.7 fold change (p=0.0719) in *mtl-2* expression (Table 34). At the highest concentration of thyme, 1.0% (v/v), *mtl-2* was lowered by 0.7 fold, which was also significantly (p=0.0000) lower than controls (Table 34).

Table 34: Effect of Different Lamiaceae Herbs on *mtl-2* (Aß peptides) Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		CL2120 (mtl-2::GFP)	
Concentration % (v/v)		FC-RF	*P* value
Basil	0.1	0.8	0.0007†
	0.5	0.8	0.0004†
	1.0	0.6	0.0000†
Oregano	0.1	1.0	0.8078
	0.5	0.7	0.0182†
	1.0	0.8	0.0001†
Rosemary	0.1	0.9	0.4358
	0.5	0.8	0.0127†
	1.0	0.7	0.0274†
Sage	0.1	0.4	0.0200†
	0.5	0.6	0.0749
	1.0	0.5	0.0812
Thyme	0.1	0.9	0.0146†
	0.5	0.8	0.0719
	1.0	0.7	0.0000†

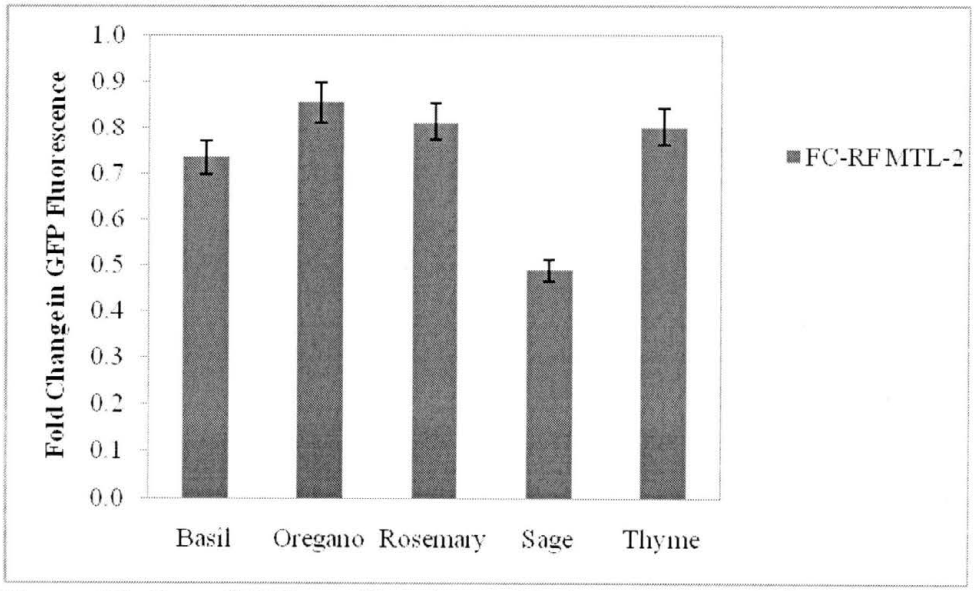

Figure 28: Overall Effect of Lamiaceae Herbs on MTL-2 (Aß) Expression in *C. elegans*. Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Metallothionein-2 (MTL-2) in the strain CL2122

CL2122 also expresses GFP which is bound to the promoter of *mtl-2*. However, this particular strain does not express human amyloid beta peptides.

After treatment with basil, *mtl-2* expression remained relatively unchanged. At 0.1% (v/v) of basil, there was a 1.1 fold change (p=0.5773) observed (Table 35). The concentration of basil at 0.5% (v/v) resulted in a 0.8 fold decrease in *mtl-2* expression, which was significantly (p=0.0032) lower than controls (Table 35). *mtl-2* expresssion after treatment with basil at 1.0% (v/v) was 1.0 fold lower than controls, however it was not significantly (p=0.4688) different.

In *C. elegans* treated with oregano, there was a significant decrease in *mtl-2* expression when compared to controls. Oregano at concentrations of 0.1% (v/v) and 0.5% (v/v) both resulted in 0.8 fold (p=0.0045; p=0.0263) decrease in *mtl-2* expression (Table 35). After treatment with 1.0% (v/v) of oregano, *mtl-2* expression was 0.6 fold lower, which was significantly (p=0.0003) decreased when compared to controls (Table 35).

Worms treated with rosemary exhibited significant increases in the expression of the MTL-2 gene. Rosemary at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in 1.3 fold (p=0.0050), 1.4 fold (p=0.0008), and 1.3 fold (p=0.0416) increase in *mtl-2* expression, respectively (Table 35).

Worms propagated on culture plates supplemented with sage or thyme had significantly less *mtl-2* expression than controls which were on NGM (Table 35). For *C. elegans* treated with sage at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) over the life cycle, *mtl-2* expression was downregulated 0.7 fold (p=0.0053), 0.6 fold (p=0.0000), and 0.7

fold (p=0.0067) of the control, respectively (Table 35). Similarly, treatment with thyme resulted in downregulation of *mtl-2* by 0.6 fold (p=0.0001), 0.6 fold (p=0.0015), and 0.7 fold (p=0.0218) of the control in worms propagated on 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) of the herb, respectively (Table 35).

Table 35: Effect of Different Lamiaceae Herbs on *mtl-2* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		CL2122 (mtl-2::GFP)	
Concentration % (v/v)		FC-RF	*P* value
Basil	0.1	1.1	0.5773
	0.5	0.8	0.0032†
	1.0	1.0	0.4688
Oregano	0.1	0.8	0.0045†
	0.5	0.8	0.0263†
	1.0	0.6	0.0003†
Rosemary	0.1	1.3	0.0050*
	0.5	1.4	0.0008*
	1.0	1.3	0.0416*
Sage	0.1	0.7	0.0053†
	0.5	0.6	0.0000†
	1.0	0.7	0.0067†
Thyme	0.1	0.6	0.0001†
	0.5	0.6	0.0015†
	1.0	0.7	0.0218†

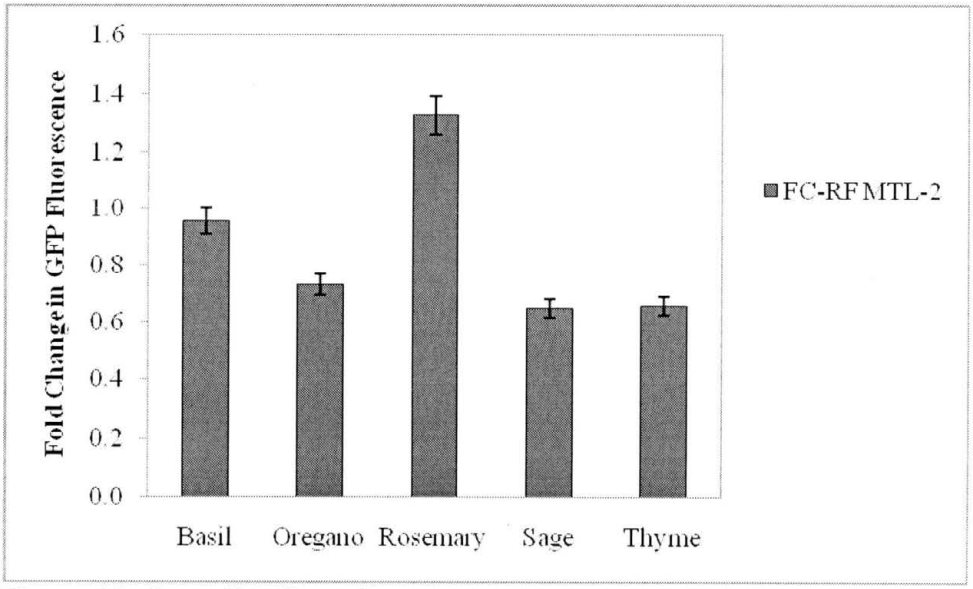

Figure 29: Overall Effect of Lamiaceae Herbs on MTL-2 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Heat shock protein-16.2 (HSP-16.2) in the strain CL2070

CL2070 expresses GFP under the control of the promoter for the inducible heat shock protein gene, *hsp-16.2* (Link et al., 1999). These proteins are most often expressed in response to thermal stress or oxidative stress (Strayer et al., 2003). However, recent work with *C.elegans* has shown that induction of heat shock proteins can occur when these organisms are exposed to heavy metals, paraquat, and other chemicals known to induce oxidative stress (Jones et al., 1996; Link et al., 1999). Previous studies have shown *hsp-16.2* is upregulated in response to reduced insulin-like signaling (Iser and Wolkow, 2007). Interestingly, exposure to thermal stress leads to rapid nuclear localization of DAF-16 and subsequent increases in heat shock proteins (Henderson and Johnson, 2001; Olsen et al., 2006).

Treatment with basil, for the most part, significantly increased *hsp-16.2* expression (Table 36). For worms feeding on 0.1% (v/v) basil, there was a 1.0 fold change, however it was not significantly (p=0.6507) different from controls (Table 36). Treatment with 0.5% (v/v) and 1.0% (v/v) basil resulted in a significant increase in *hsp-16.2* by 1.1 fold (p=0.0098) and 1.1 fold (p=0.0096), respectively (Table 36).

Expression of *hsp-16.2* remained relatively unchanged after treatment with oregano. At the lowest concentration of oregano, 0.1% (v/v), there was a 1.0 fold decrease is gene expression, however it was not significantly (p=0.4828) different from worms on NGM (Table 36). Additionally, treatment with 0.5% (v/v) of oregano resulted in a significant (p=0.0003) 0.9 fold decrease in *hsp-16.2* expression (Table 36). *hsp-16.2* expression increased by 1.1 fold after treatment with oregano at 1.0% (v/v), however it was not significantly (p=0.4977) different from the controls (Table 36).

A similar effect in expression of *hsp-16 2* was observed after treatment with rosemary. There was a 1.0 fold change (p=0.9019) in *hsp-16 2* expression in worms feeding on 0.1% (v/v) rosemary (Table 36). For *C. elegans* feeding on 0.5% (v/v) of rosemary, there was a significant (p=0.0146) 0.9 fold decrease in *hsp-16.2* expression when compared to controls (Table 36). Treatment with rosemary at the highest concentration, 1.0% (v/v), resulted in a 1.1 fold increase in gene expression, however it was not significantly (p=0.2063) different from controls (Table 36).

For worms feeding on 0.1% (v/v) and 0.5% (v/v) sage, there was a significant decrease by 0.8 fold (p=0.0008) and 0.9 fold (p=0.0397) in *hsp-16.2* expression, respectively (Table 36). However, expression of *hsp-16.2* was significantly (p=0.0370) higher than controls by 2.7 fold in worms treated with 1.0% (v/v) of sage (Table 36).

It was observed that, for the most part, worms propagated on culture plates supplemented thyme had significant decreases in *hsp-16.2* expression. Thyme at 0.1% (v/v) resulted in a 0.8 fold decrease (p=0.0656) in gene expression (Table 36). *hsp-16.2* expression after treatment with thyme at 0.5% (v/v) and 1.0% (v/v) was significantly (p=0.0011; p=0.0015) decreased by 0.5 fold, respectively (Table 36).

Table 36: Effect of Different Lamiaceae Herbs on *hsp-16.2* Expression in *C. elegans*. Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

Concentration % (v/v)		CL2070 (hsp-16.2::GFP)	
		FC-RF	*P* value
Basil	0.1	1.0	0.6507
	0.5	1.1	0.0098*
	1.0	1.1	0.0096*
Oregano	0.1	1.0	0.4828
	0.5	0.9	0.0003†
	1.0	1.1	0.4977
Rosemary	0.1	1.0	0.9019
	0.5	0.9	0.0146†
	1.0	1.2	0.2063
Sage	0.1	0.8	0.0008†
	0.5	0.9	0.0397†
	1.0	2.7	0.0370*
Thyme	0.1	0.8	0.0656
	0.5	0.5	0.0011†
	1.0	0.5	0.0015†

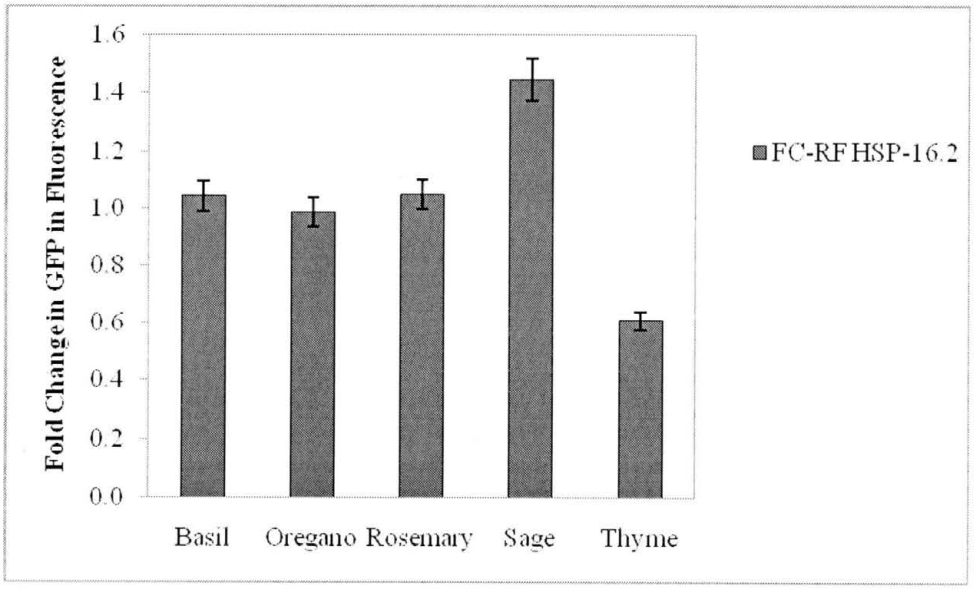

Figure 30: Overall Effect of Lamiaceae Herbs on HSP-16.2 Expression in *C. elegans*. Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Iron sulphur protein-1 (ISP-1) in the strain BC14279

The transgenic strain, BC14279, has GFP bound to the promoter of *isp-1*, the gene which encodes an iron sulphur protein (ISP). This ISP is a subunit of the mitochondrial complex III, and transcription of this protein is under regulation of the transcription factor, SKN-1/Nrf2 (Baumeister et al., 2006).

It was observed that treatment with basil did not significantly change expression of *isp-1*. Gene expression at 0.1% (v/v), 0.5% (v/v), 1.0% (v/v) of basil was 0.5 fold (p=0.0917), 0.5 fold (p=0.0799), and 1.0 fold (p=0.4874) different from controls, respectively (Table 37).

Similarly, worms propagated on plates supplemented with oregano did not exhibit significant changes in *isp-1* expression. Treatment with 0.1% (v/v) and 0.5% (v/v) of oregano resulted in a 1.2 fold (p=0.1027) and 1.6 fold (p=0.1809) increase in *isp-1* expression, respectively (Table 37). However, oregano at 1.0% (v/v) resulted in a significant (p=0.0226) 1.4 fold increase in gene expression (Table 37).

In *C. elegans* feeding on rosemary plates, the highest increases in *isp-1* expression were observed. *isp-1* expression after treatment with 0.1% (v/v) of rosemary significantly (p=0.0002) increased 3.1 fold when compared to controls (Table 37). Rosemary at 0.5% (v/v) of also resulted in a 3.1 fold increase, however it was not significantly (p=0.1055) different from worms on NGM (Table 37). At the highest concentration of rosemary, 1.0% (v/v), there was a 2.4 fold increase in *isp-1* expression, however it was not significantly (p=0.0504) different from controls (Table 37).

Sage was also shown to increase *isp-1* expression on *C. elegans*. There was a 3.2 fold increase in gene expression after treatment with 0.1% (v/v) of sage, which was

significantly (p=0.0013) higher than controls (Table 37). Treatment with 0.5% (v/v) and 1.0% (v/v) of sage resulted in 1.5 fold increases in *isp-1* expression, however they were not significantly (p=0.0879; p=0.0910) different from worms on NGM (Table 37).

Worms treated with thyme had increased *isp-1* expression when compared to controls. Thyme at 0.1 % (v/v) and 0.5% (v/v) resulted in a 3.2 fold (p=0.1032) and 2.3 fold (p=0.1571) increase in *isp-1* expression, however it was not significantly different from controls (Table 37). At the highest concentration of thyme, 1.0% (v/v), there was a significant (p=0.0007) 1.2 fold increase in gene expression when compared to worms in NGM (Table 37).

Table 37: Effect of Different Lamiaceae Herbs on *isp-1* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		BC14279 (isp-1::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	0.5	0.0917
	0.5	0.5	0.0799
Basil	1.0	1.0	0.4874
	0.1	1.2	0.1027
	0.5	1.6	0.1809
Oregano	1.0	1.4	0.0226*
	0.1	3.1	0.0002*
	0.5	3.1	0.1055
Rosemary	1.0	3.1	0.0504
	0.1	3.2	0.0013*
	0.5	1.5	0.0879
Sage	1.0	1.5	0.0910
	0.1	3.2	0.1032
	0.5	2.3	0.1571
Thyme	1.0	1.2	0.0007*

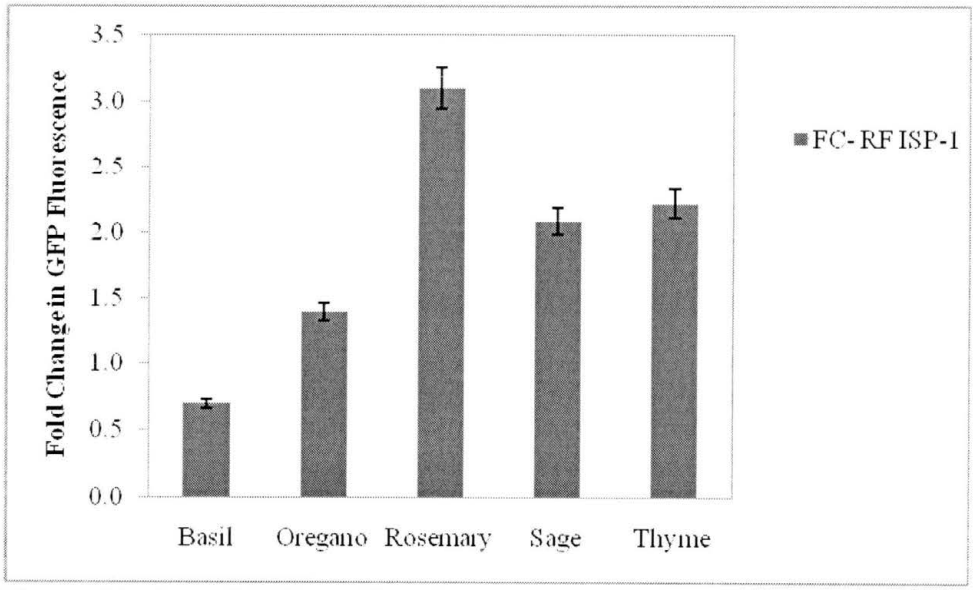

Figure 31: Overall Effect of Lamiaceae Herbs on ISP-1 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of γ-glutamine cysteine synthetase-1 (GCS-1) in the strain VC337

This particular strain contains a GFP construct bound to the promoter of *gcs-1*. This gene encodes the phase II detoxification enzyme, γ-glutamine cysteine synthetase, which is involved as a rate limiting enzyme in glutathione biosynthesis (An and Blackwell, 2003; Tullet et al., 2008). Induction of *gcs-1* expression in *C. elegans* has been shown to occur during times of oxidative stress by the transcription factor SKN-1 (An and Blackwell, 2003; Inoue et al., 2005).

Worms treated with basil had significantly higher expression of *gcs-1* when compared to worms propagated on NGM (Table 38). *gcs-1* expression in worms consuming 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) basil was significantly higher by 1.1 fold (p=0.0003), 1.1 fold (p=0.0002), and 1.2 fold (p=0.0005) than controls, respectively (Table 38).

Similarly, it was observed that an upregulation of *gcs-1* in *C. elegans* feeding on all concentrations of oregano when compared to controls. There was a 1.4 fold increase in *gcs-1* expression at 0.1% (v/v) (p=0.0001), 0.5% (v/v) (p=0.0000), and 1.0% (v/v) (p=0.0002) of oregano (Table 38).

Treatment with rosemary resulted in significant decreases in *gcs-1* expression when compared to controls. Rosemary at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in significant decreases in *gcs-1* expression by 0.7 fold (p=0.0001), 0.8 fold (p=0.0000), and 0.9 fold (p=0.0052), respectively (Table 38).

In *C. elegans* feeding on plates supplemented with sage, *gcs-1* expression was significantly higher than worms on NGM. At 0.1% (v/v) of sage, there was a 1.2 fold increase in *gcs-1* expression, which was significantly (p=0.0000) increased when

compared to controls (Table 38). *gcs-1* expression after treatment with sage at 0.5% (v/v) and 1.0% (v/v) was significantly higher than controls by 1.3 fold (p=0.0302) and 1.4 fold (p=0.0034), respectively (Table 38).

Expression of *gcs-1* remained relatively unchanged after worms were treated with thyme. At the lowest concentration of thyme, 0.1% (v/v), there was a significant (p=0.0010) 0.8 fold decrease in *gcs-1* expression when compared to controls (Table 38). However, treatment with 0.5% (v/v) and 1.0% (v/v) resulted in a 1.3 fold (p=0.4756) and 1.5 fold (p=0.2392) change, respectively, which was not significantly different from the controls (Table 38).

Table 38: Effect of Different Lamiaceae Herbs on *gcs-1* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		VC337 (gcs-1::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	1.1	0.0003*
	0.5	1.1	0.0002*
Basil	1.0	1.2	0.0005*
	0.1	1.4	0.0001*
	0.5	1.4	0.0000*
Oregano	1.0	1.4	0.0002*
	0.1	0.7	0.0001†
	0.5	0.8	0.0000†
Rosemary	1.0	0.9	0.0052†
	0.1	1.2	0.0000*
	0.5	1.3	0.0302*
Sage	1.0	1.4	0.0034*
	0.1	0.8	0.0010†
	0.5	1.3	0.4756
Thyme	1.0	1.5	0.2392

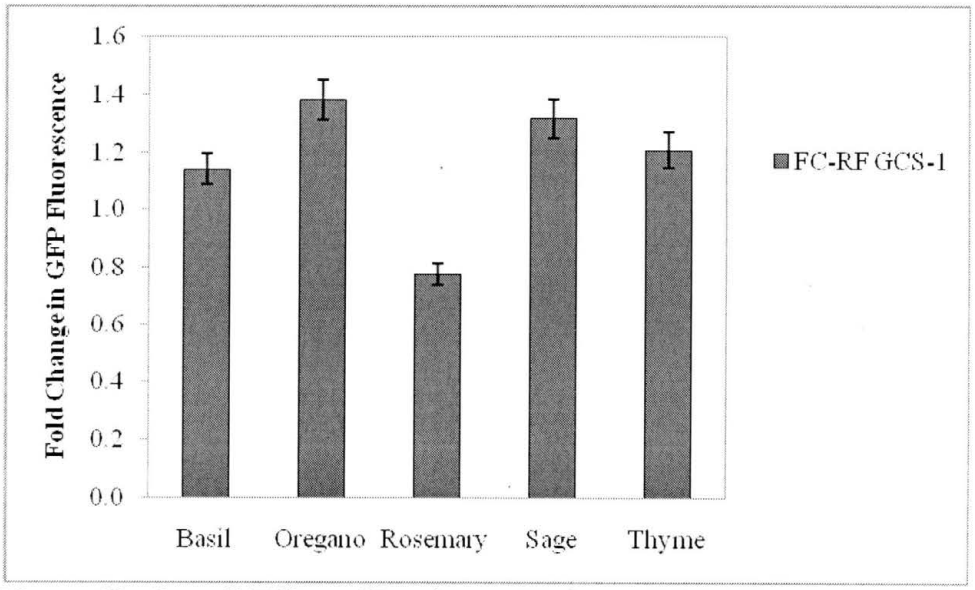

Figure 32: Overall Effect of Lamiaceae Herbs on GCS-1 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Glutathione S-Transferase-4 (GST-4) in the strain BC13348

BC13348 carries a transcriptional fusion of GFP to the *gst-4* promoter. Glutathione S-transferase is a phase II detoxification enzyme most often expressed in response to oxidative stress. Additionally, previous studies have indicated that expression of *gst-4* is under the regulation of SKN-1 (An and Blackwell, 2003; Kell at el, 2007).

It was shown that expression of *gst-4* was significantly increased in response to treatment with basil when compared to controls. For *C.elegans* feeding on basil at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) there was a significant increase in *gst-4* expression by 1.1 fold (p=0.0349), 1.2 fold (p=0.0005), and 1.1 fold (p=0.0002), respectively, when compared to controls (Table 39).

Expression of *gst-4* was not significantly different between worms feeding on oregano and the controls which were only on NGM. However, there was a 1.0 fold change in *gst-4* expression for all concentrations of oregano; yet it was not significantly different from controls (Table 39).

For the most part, treatment with rosemary also did not significantly change *gst-4* expression when compared to controls. At 0.1% (v/v) and 0.5% (v/v) of rosemary, *gst-4* expression changed 1.0 fold, however it was not significantly (p=0.7725; p=0.1243) different from worms on NGM (Table 39). *gst-4* expression was significantly lower by 1.0 fold (p=0.0166) for worms propagated on 1.0% (v/v) of rosemary (Table 39).

In *C. elegans* feeding on 0.1% (v/v) and 0.5% (v/v) of sage, there was a significant increase in *gst-4* expression by 1.1 fold (p=0.0001) and 1.0 fold (p=0.0019), respectively (Table 39). However at the highest concentration of sage, 1.0% (v/v), there

was a 1.0 fold decrease in *gst-4* expression, but it was not significantly (p=0.6937) different from controls (Table 39).

It was observed that *gst-4* expression, for the most part, after treatment with thyme was not significantly different from controls. *gst-4* expression was decreased by 1.0 fold for worms on 0.1% (v/v) (p=0.5715) and 0.5% (v/v) (p=0.6268) thyme (Table 39). However thyme at 1.0% (v/v), resulted in a significant (p=0.0005) 1.0 fold increase in *gst-4* expression when compared to worms on NGM (Table 39).

Table 39: Effect of Different Lamiaceae Herbs on *gst-4* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		BC13348 (gst-4::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	1.1	0.0349*
	0.5	1.2	0.0005*
Basil	1.0	1.1	0.0002*
	0.1	1.0	0.9174
	0.5	1.0	0.3817
Oregano	1.0	1.0	0.9892
	0.1	1.0	0.7725
	0.5	1.0	0.1243
Rosemary	1.0	1.0	0.0166†
	0.1	1.1	0.0001*
	0.5	1.0	0.0019*
Sage	1.0	1.0	0.6937
	0.1	1.0	0.5715
	0.5	1.0	0.6268
Thyme	1.0	1.0	0.0005*

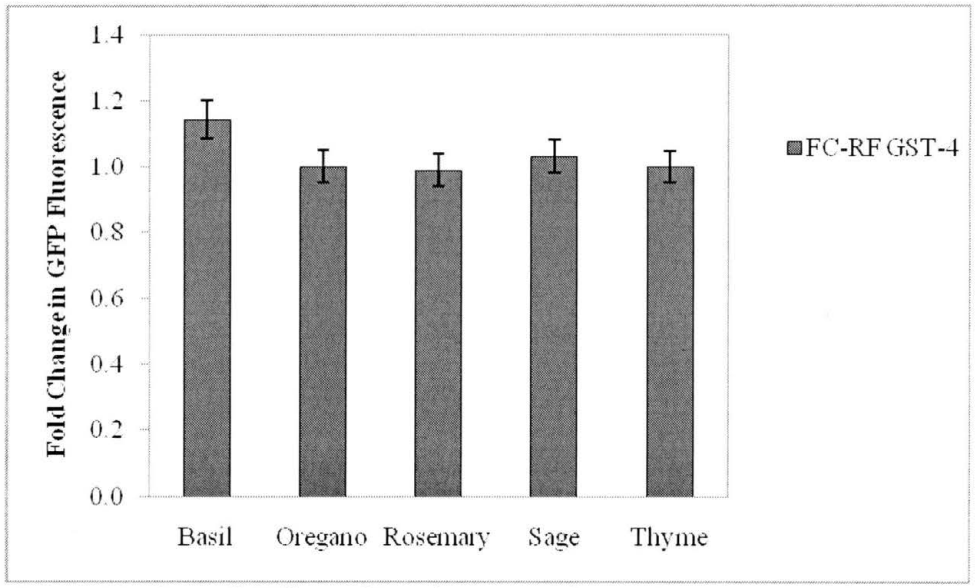

Figure 33: Overall Effect of Lamiaceae Herbs on GST-4 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Erythroid-Like Transcription Factor-2 (ELT-2) in the strain OH7631

OH7631 carries a transcriptional fusion of GFP to the promoter of *elt-2*. ELT-2 is a gut specific transcription factor important for the development of the gut during embryogenesis and activation of gut specific genes such as those for metallothioneins (Freedman et al., 1993; Fukushige et al., 1998). Activation of *elt-2* occurs early in embryogenesis through binding of maternal SKN-1 to the promoter of the gene (Fukushige et al., 1998).

The data indicates that *elt-2* expression was relatively unchanged after treatment with basil. There was a 0.7 fold decrease in *elt-2* expression after treatment with 0.1% (v/v) of basil, however it was not significantly (p=0.2509) different from controls (Table 40). Basil at 0.5% (v/v) resulted in a significant (p=0.337) 0.6 fold decrease in *elt-2* expression (Table 38). At the highest concentration of basil, 1.0% (v/v), *elt-2* expression was decreased by 0.4 fold, but was not significantly (p=0.0601) from controls (Table 40).

Similarly, worms propagated on oregano plates had relatively similar *elt-2* expression when compared to controls. Treatment with oregano at 0.1% (v/v) and 0.5% (v/v) resulted in a 0.6 fold (p=0.1322) and 0.7 fold (p=0.3717) decrease in *elt-2*, respectively (Table 40). Treatment at 1.0% (v/v) of basil resulted in a 0.5 fold decrease in *elt-2* expression, which was significantly (p=0.0478) lower than controls (Table 40).

In *C. elegans* feeding on rosemary, there were significant decreases in *elt-2* expression when compared to controls. Gene expression at 0.1% (v/v) of rosemary was 1.1 fold higher than controls, however there was not a significant (p=0.4942) difference between the two treatments (Table 40). Treatment with rosemary at 0.5% (v/v) and 1.0%

(v/v) resulted in significant (p=0.0433; p=0.0384) decreases in *elt-2* expression by 0.5 fold (Table 40).

On the other hand, treatment with sage resulted in significant upregulation of *elt-2* expression when compared to controls. At the lowest concentration of sage, 0.1% (v/v), there was a 1.2 fold increase in gene expression, however it was not significantly (p=0.3200) different from controls (Table 40). In contrast, *elt-2* expression was significantly increased by 1.4 fold (p=0.0205) and 1.7 fold (p=0.0052) after treatment with 0.5% (v/v) and 1.0% (v/v) of sage, respectively (Table 40).

Expression of *elt-2* remained unchanged after treatment with thyme when compared to controls. At 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) of thyme there was a 0.9 fold (p=0.5237) decrease, 1.0 fold (p=0.4469) increase, 1.0 fold decrease (p=0.9106) in *elt-2* expression, respectively, however there was no significant difference when compared to controls (Table 40).

Table 40: Effect of Different Lamiaceae Herbs on *elt-2* Expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		OH7631 (elt-2::GFP)	
Concentration % (v/v)		FC-RF	*P* value
	0.1	0.7	0.2509
	0.5	0.6	0.0337†
Basil	1.0	0.4	0.0601
	0.1	0.6	0.1322
	0.5	0.7	0.3717
Oregano	1.0	0.5	0.0478†
	0.1	1.1	0.4942
	0.5	0.5	0.0433†
Rosemary	1.0	0.5	0.0384†
	0.1	1.2	0.3200
	0.5	1.4	0.0205*
Sage	1.0	1.7	0.0052*
	0.1	0.9	0.5237
	0.5	1.0	0.4469
Thyme	1.0	1.0	0.9106

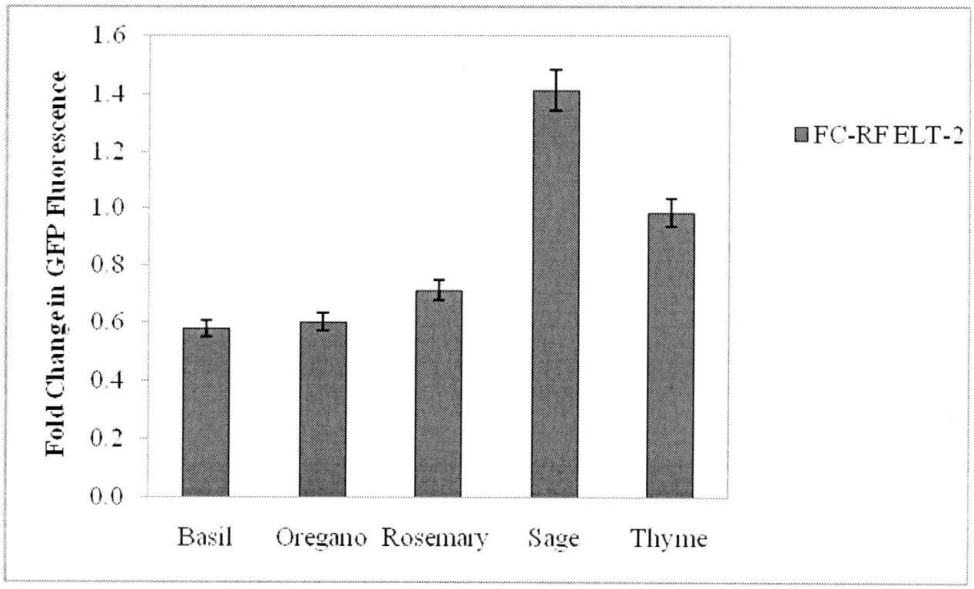

Figure 34: Overall Effect of Lamiaceae Herbs on ELT-2 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Effect of Lamiaceae herbs on expression of Forkhead Transcription Factor-6 (FKH-6) in the strain DZ325

DZ325 has an integrated GFP promoter construct bound to *fkh-6*. FKH-6 is a transcription factor specific to the cells of the gonad in *C. elegans,* where it functions as a regulator of sexual dimorphism (Chang et al., 2004). In *fkh-6* mutant males the gonads were feminized and resembled that of hermaphrodites (Chang et al., 2004).

It was observed that treatment with basil, for the most part, significantly increased expression of *fkh-6* when compared to controls. At the lowest concentration of basil, 0.1% (v/v), there was a 1.0 fold increase in *fkh-6* expression, however it was not significantly (p=0.2705) different from the worms on NGM (Table 40). Expression of *fkh-6* was significantly increased by 1.2 fold (p=0.0108) and 1.3 fold (p=0.0005) after treatment with 0.5% (v/v) and 1.0% (v/v) of basil, respectively (Table 41).

In *C. elegans* treated with oregano there was not a significant change in *fkh-6* expression when compared with controls. There was 1.0 fold (p=0.5105) increase, 1.1 fold (p=0.3217) increase, and a 1.0 fold (p=0.2044) decrease in gene expression after treatment with 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) of oregano, respectively (Table 41).

For worms feeding on 0.1% (v/v) of rosemary, *fkh-6* expression was significantly (p=0.0426) increased by 1.1 fold when compared to worms on NGM (Table 41). However, rosemary at 0.5% (v/v) and 1.0% (v/v) resulted in a decrease in *fkh-6* expression by 1.0 fold which was not significantly different from controls (Table 41).

Similarly, *fkh-6* expression was not significantly different from controls after treatment with sage. Treatment with 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) of sage

resulted in a 1.0 fold (p=0.2965) increase, 1.1 fold (p=0.1649), and a 1.1 fold (p=0.2701) increase in *fkh-6* expression, respectively (Table 41).

It was observed that worms propagated on culture plates supplemented with thyme had significantly higher *fkh-6* expression than controls. Treatment with thyme at 0.1% (v/v), 0.5% (v/v), and 1.0% (v/v) resulted in significant increases in *fkh-6* expression by 1.2 fold (p=0.0244), 1.5 fold (p=0.0000), and 1.4 fold (p=0.0028), respectively (Table 41).

Table 41: Effect of different Lamiaceae herbs on *fkh-6* expression in *C. elegans*.
Relative change in fluorescence intensity in *C. elegans* treated with different Lamiaceae herb extracts at concentrations of 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) compared to control, as measured using the Nikon SMZ1500 fluorescence microscope, Ri1 CCD camera for imaging and NIH ImagJ software for quantification. N=6. Fold Change-Relative Fluorescence (FC-RF)
*-indicates Treatment + NGM is significantly higher than NGM ($p<0.05$)
†-indicates Treatment + NGM is significantly lower than NGM ($p<0.05$)

		DZ325 (fkh-6::GFP)	
Concentration % (v/v)		FC-RF	*P* value
Basil	0.1	1.0	0.2705
	0.5	1.2	0.0108*
	1.0	1.3	0.0005*
Oregano	0.1	1.0	0.5105
	0.5	1.1	0.3217
	1.0	1.0	0.2044
Rosemary	0.1	1.1	0.0426*
	0.5	1.0	0.7942
	1.0	1.0	0.4923
Sage	0.1	1.0	0.2965
	0.5	1.1	0.1649
	1.0	1.1	0.2701
Thyme	0.1	1.2	0.0244*
	0.5	1.5	0.0000*
	1.0	1.4	0.0028*

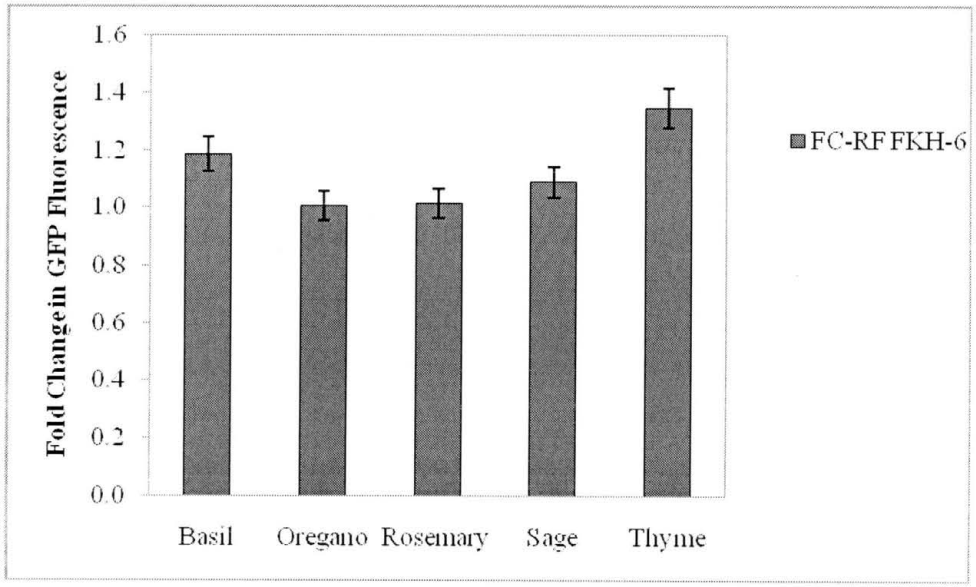

Figure 35: Overall Effect of Lamiaceae Herbs on FKH-6 Expression in *C. elegans*.
Average fold change in relative fluorescence intensity in *C. elegans* compared to control

Discussion

One of the best characterized and intensely studied pathways in *C. elegans* involves signaling through the insulin/insulin-like growth factor-I (IGF-I) receptor, DAF-2. Signaling through this receptor is initiated through the binding of an insulin-like molecule to the DAF-2 receptor, and subsequent activation of the conserved phosphoinositide-3-kinase (PI3K) pathway (Gami and Wolkow, 2006; Greer and Brunet, 2008). Emerging evidence suggest that the organism's ability to manage oxidative stress and alter metabolic processes, which ultimately affects lifespan, occurs through regulation of DAF-16, a FOXO transcription factor homolog (Libina et al., 2003; Gami et al., 2006) It has been shown that an active insulin-like pathway phosphorylates DAF-16, thereby inhibiting its nuclear translocation and ability to initiate transcription of target genes. Several studies have identified potential downstream genes of DAF-16 which could possibly explain an increased resistance to stress and longevity in strains of *C. elegans* which have lowered insulin-like signaling (Murphy et al., 2003; Murphy, 2006). In these strains, genes linked to growth and development are typically downregulated, while genes linked to stress resistance such as superoxide dismutase (*sod-3*), catalase *(ctl-1,-2,-3*), and heat shock proteins (*hsp-16.2*) are upregulated (Figure 36). Scientists have also identified another transcription factor that functions to control the expression of genes involved in the stress response in *C. elegans*. SKN-1, a protein homologous to mammalian Nrf1 and Nrf2, initiates transcription of genes such as *gst-4*, *gcs-1*, *isp-1,* and *elt-2* (Inoue et al., 2005; Tullet et al., 2008) (Figure 36). In times of oxidative stress, an active p38 MAPK pathway promotes the nuclear accumulation of SKN-1 and transcription of genes important for stress resistance and longevity (Inoue et al., 2005;

Baumeister et al., 2006) However under normal conditions, SKN-1 is phosphorylated by glycogen synthase kinase (GSK-3) and retained within the cytosol of intestinal cells (An et al., 2005) (Figure 36). Interestingly, many of these genes in *C. elegans* have homologs in the vertebrate genome. The conservation of genes among various organisms and similarity in signaling pathways allows researchers to elucidate potential mechanisms of action for disease development and prevention.

In this study, *Caenorhabditis elegans* was used as a model organism to understand the mechanism of redox modulation by Lamiaceae herbs. Given the importance of the insulin-like signaling pathway in the regulation of genes responsible for resistance to oxidative stress, the current study quantified expression of genes downstream of the DAF-2/insulin-like receptor. Overall, it was observed that Lamiaceae herbs exhibited variable effects on gene expression. This variation may be attributed to the unique phytochemical profile that each herb possesses.

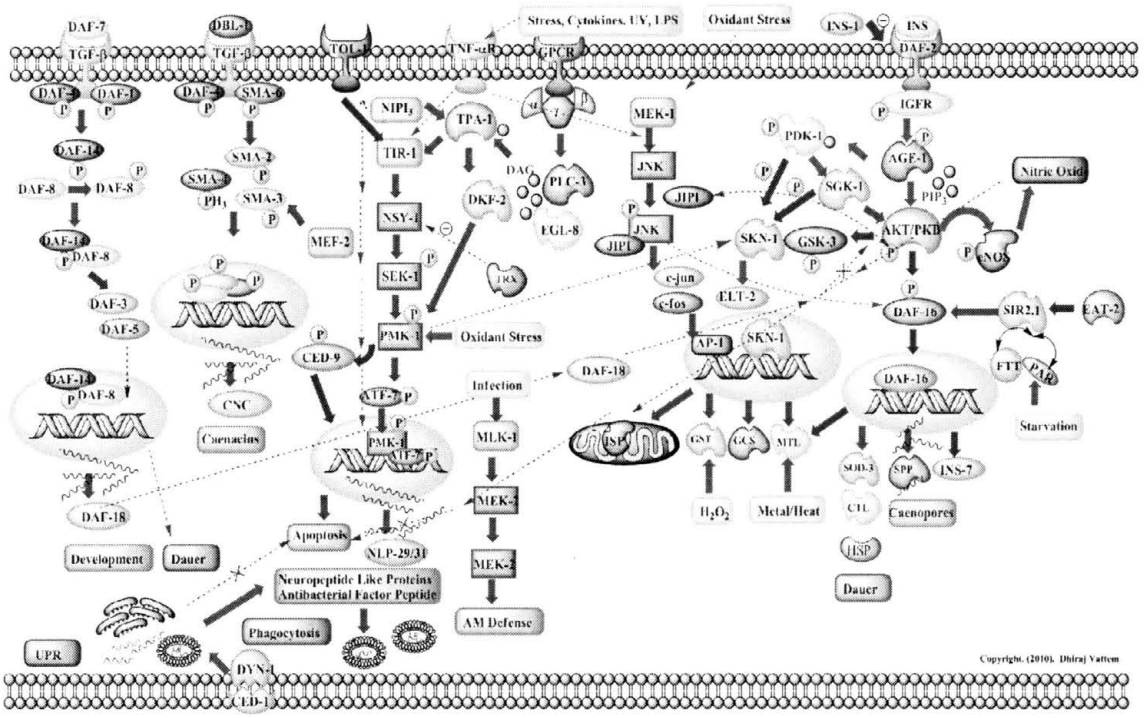

Figure 36: Various Stress Response Pathways in *C. elegans*.

In the present study, the data revealed that treatment with basil significantly increased expression of *daf-16* in the transgenic strains, TJ356 and GR1352, when compared to controls which were on a standard growth medium. The transgenic strains, CF1553 (*sod-3)*, GA800 (*ctl-1,-2,-3)*, CL2070 (*hsp-16.2*), CL2120 (*mtl-2*), and CL2122 (*mtl-2*), were used to measure expression of genes under the transcriptional regulation of DAF-16 (Figure 36). Interestingly, it was observed that there was a significant increase in *ctl-1,-2,-3* expression by 1.3 fold and significant increases *hsp-16.2* by 1.0 fold after treatment with basil. It was also observed that expression of *mtl-2*, which is negatively regulated by DAF-16, was significantly downregulated after treatment with basil by 0.7 fold. The nuclear translocation of DAF-16 has been shown to increase expression of *sod-3*, *ctl-1,-2,-3*, *hsp-16.2*, and down regulate *mtl-2* (Murphy et al., 2003). The results

suggest that treatment with basil increased expression and nuclear translocation of DAF-16, resulting in transcriptional activities favoring the synthesis of catalase, SOD, and heatshock proteins (Figure 36). These results are consistent with a previous study where Weigant et al. indicated that treatment with the natural products, *Rhodiola rosea* and *Eleuthoerococcus senticosus*, promoted the nuclear translocation of DAF-16 and subsequently increased stress resistance (Wiegant et al., 2009). In yet another study with herbal supplements, Yu et al. have also shown that treatment with, *Cinnamomum cassia* and *Panax ginseng*, modulated expression of genes involved in insulin signaling and increased expression of the stress response gene, *hsp-16.2* (Yu et al., 2010). In addition to measuring genes under regulation of DAF-16, genes under transcriptional regulation of SKN-1 were measured. There were no significant differences in expression of *elt-2* and *isp-1* between the treatment group and the controls. However, there were significant increases in the expression of *gcs-1* and *gst-4* by 1.1 fold when compared to controls. These results suggest that SKN-1 was able to translocate to the nucleus and inititate transcription of phase II enzymes. This was shown to be the case in a study regarding an increased stress response and longevity in quercetin treated *C. elegans*. In this study, Pietsch et al. suggested altered signaling via the p38 MAPK pathway as the mechanism by which SKN-1 was able to initiate transcription of stress resistance genes (Pietsch et al., 2009) (Figure 36). This may be a possible mechanism by which phase II enzymes in the current study were increased as well. In future studies mutant strains of *C. elegans* for proteins within this pathway will be utilized as well as RNA interference, to identify a potential mechanism of action. In addition to previous work in *L. terrestris* where it was observed that there were increases in the ratio of healthy /deformed sperm and

mature/immature sperm, in the current study expression of *fkh-6* was measured. This gene codes for a transcription factor that is essential for the development of the male gonad in *C. elegans*. Interestingly, the data revealed an average 1.2 fold increase in gene expression when compared to controls. This provides further evidence of the potential that Lamiaceae herbs have in altering spermatogensis and reproductive development.

Treatment with oregano did not result in significant changes in expression of *daf-16* as did basil. The downstream genes regulated by DAF-16, *sod-3, ctl-1,-2,-3*, and *hsp-16.2*, remained relatively unchanged in response to treatment as well. However, expression of *mtl-2* in the transgenic strains, CL2120 and CL2122, was downregulated. Interestingly, the transcription factor *elt-2* has been shown to induce expression of *mtl-2* (Moilanen et al., 1999) (Figure 36). It was found that *elt-2* expression was significantly downregulated at the highest concentration of oregano, and expression was decreased with the lower concentrations however it was not significant. The expression of genes under regulation of SKN-1, *isp-1* and *gst-4*, on average, were not significantly different from the controls. However, *gcs-1* expression was significantly increased in oregano treated worms when compared to controls. Overall, it was observed that expression of genes downstream of the insulin receptor remained relatively unchanged in comparison to controls. Previous research has indicated that an active insulin-like signaling pathway leads to cytosolic rentention of both SKN-1 and DAF-16 (Lee et al., 2001; Murphy et al., 2003; Tullet et al., 2008). AKT-1,-2 and SGK-1 phosphorylate SKN-1 at multiple sites, similar to DAF-16, preventing nuclear accumulation (Tullet et al., 2008) (Figure 36). This could possibly be the mechanism by which genes under regulation of these transcription factors remained relatively unchanged. However, future research with

mutants containing deletions of proteins upstream of the target genes studied will be needed in order to confirm this proposed mechanism of action. In addition, measurements of *fkh-6* expression revealed that treatment with oregano did not significantly increase expression.

Treatment with rosemary had effects similar to that of oregano. Expression of *daf-16,* on average, was not significantly different from the control group. It was found that genes under regulation of DAF-16, for the most part, were significantly down regulated after treatment with rosemary. For the genes under transcriptional regulation of SKN-1, there was an observed downregulation of *gcs-1, gst-4,* and *elt-2* (Figure 36). Expression of *isp-1*, which is also under regulation of SKN-1, on average, was not significantly different from the control. These results suggest active *daf-2* signaling resulting in phosphorylation of DAF-16 and SKN-1, preventing transcription of target genes. It was also observed that significant increases in *daf-9* expression after treatment with rosemary. Interestingly, DAF-9 is the point of convergence downstream of DAF-2 and DAF-7, which is involved in the regulation of dauer formation (Jia et al., 2002). In addition to genes under SKN-1 and DAF-16, the data also revealed no significant changes in *fkh-6* expression when compared to controls.

Upon evalution of gene expression after treatment with sage, it appears as if this herb had effects similar to that of basil. In one of the transgenic strains of *daf-16*, TJ356, there were not significant changes in expression after treatment with sage. However in GR1352, *daf-16α*, there were significant increases in expression at all concentrations used. When expression of *sod-3* and *ctl-1,-2,-3* were measured, there were no significant changes in expression compared to controls. The expression of *hsp-16.2* was also

variable, with the lowest concentrations resulting in significant decreases in expression and the highest concentration resulting in a significant increase. Previous work on DAF-16 suggests that a reduction in insulin-like signaling isn't always associated with transcription of target genes. It is believed that a cofactor, yet to be identified, is needed to activate DAF-16 (Hertweck et al., 2004; Baumeister et al., 2006). This may be a possible explanation for the observations that were made after treatment with sage, where it was indicated that there were significant increases in *daf-16* expression, but not in the genes downstream of DAF-16. For the genes under regulation of SKN-1, the data revealed significant increases in expression of *gcs-1*, *gst-4, elt-2,* and *isp-1*. As was the case with in worms treated with basil, treatments with sage may have altered signaling via the p38 MAPK pathway allowing for the nuclear translocation of SKN-1 (Figure 36). There may have been reductions in insulin-like signaling as well, as it was observed that after treatment with sage *daf-9* expression, a downstream target of insulin signaling, was lower in comparison to control but not significantly different. Additionally, evaluations of the transcription factor necessary for male gonadal development, *fkh-6*, revealed no significant changes in expression after treatment.

In *C. elegans* treated with thyme, it was observed that there were significant decreases in *daf-16* expression. Also the genes under regulation of DAF-16, *sod-3* and *ctl-1,-2,-3*, were downregulated as well; however not significantly different from controls. On the other hand, the expression of heatshock proteins was significantly downregulated in the sage treatment group when compared to controls. For genes under the regulation of SKN-1, *isp-1, gst-4, gcs-1*,and *elt-2,* there was relatively no significant changes in expression observed when compared to controls. Also metallothionein, which

is under transcriptional regulation of *elt-2* was significantly decreased. These results suggest active *daf-2*/insulin-like signaling, leading phosphorylation of the transcription factors, SKN-1 and DAF-16. Another indication of an active insulin-like signaling pathway is that the downstream target of *daf-2* signaling, *daf-9*, was significantly increased in thyme treated *C. elegans*. In addition to previous work in *L. terrestris* where it was observed that there were increases in the ratio of healthy /deformed sperm and mature/immature sperm after treatment with thyme, it was observed that there was a significant 1.3 fold increase in *fkh-6* gene expression when compared to controls.

Conclusion

In Chapter 1, work with *Lumbricus terrestris* revealed significant increases in antioxidant enzyme activity and nitric oxide metabolites in response to a 6 day treatment with different Lamiaceae herbs. Additionally, there were significant decreases in lipid oxidation, sperm deformations, and DNA fragmentation. Data analysis suggested modulation of redox response via protein kinase C, ARE-Nrf2, and SP-1/AP-2 mediated expression of SOD, glutathione peroxidase and nitric oxide synthase. In Chapter 2, the organism was in a state of peroxide induced oxidative stress, and data revealed that treatment with these herbs restored redox homeostatis. In order to identify the genetic effects of treatment with Lamiaceae herbs *Caenorhabditis elegans*, an established model for studying molecular signaling, was utilized to monitor changes in gene expression of proteins relevant to the insulin-like signaling pathway. Similar to the previous study with *Lumbricus terrestris,* the work with *Caenorhabditis elegans* revealed that herbs from the same family can have different effects on redox modulaton. These differences could possibly be attributed to the variation in both phytochemical content and profile among

plants which belong to the same family (Kivilompolo and Hyotylainen, 2007). In summary of the work with *C. elegans*, it was observed that treatment with basil and sage had similar effects in that they may have altered signaling of the p38 MAPK pathway, promoting nuclear accumulation of SKN-1 and transcription of target genes. It may also be possible that there was reduced insulin-like signaling, as the data revealed increases in expression of genes negatively regulated by this pathway. An opposite effect was observed in worms treated with oregano, rosemary, and thyme. The data indicated that there were reductions in the expression of *daf-16* and its target genes, therefore suggesting an active insulin-like pathway. In addition, expression of genes under regulation of SKN-1 were downregulated as well. The results from this study provide some information about a potential mechanism of action by which Lamiaceae herbs are able to alter redox status *in vivo*. For future studies we will utilize genetic knockouts and RNA interference to silence genes within these pathways to confirm what was observed in the current body of work.

Abbreviations

AB	Amyloid beta peptide
AUC	Area under the curve
AOX	Antioxidant
ARE	Antioxidant response elements
ATP	Adenosine triphosphate
BA	Basil
BSA	Bovine serum albumin
CAT	Catalase
CTL	Catalase
CVD	Cardiovascular disease
DFO	Sperm deformations
DNA	Deoxyribonucleic acid
DPA	Diphenyl amine
EDTA	Ethylenediaminetetraacetic acid

ELT	Erythroid-like transcription factor
EST	Expressed sequence tag
ETC	Electron transport chain
Fe	Iron
FOXO	Forkhead box transcription factor
FKH	Forkhead transcription factor
GCS	γ- glutamyl cysteine synthetase
GFP	Green fluorescent protein
GI	Gastrointestinal
GPx	Glutathione peroxidase
GSH	Reduced Glutathione
GST	Glutathione S-transferase
4-HNE	4-hydroxynonenal
HSP	Heatshock protein
IGF	Insulin like growth factor
IKK	I kappa kinase
ISP	Iron sulphur protein
LDL	Low density lipoprotein
LBSS	Lumbricus balanced salt solution
LGM	*Lumbricus* growth medium
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
Mn	Manganese
MTL	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NF-κB	Nuclear factor-kappa B
NGM	Nematode growth medium
NO	Nitric oxide
NOS	Nitric oxide synthase
OR	Oregano
PCR	Polymerase chain reaction
PER	Peroxide
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
SOD	Superoxide dismutase
TH	Thyme

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