ANTI-NEURODEGENERATIVE EFFECTS OF SAGE (*SALVIA OFFICINALIS*) VIA MODULATION OF STRESS RESPONSE SIGNALING IN *C. ELEGANS*

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CHAPTER I

DIETARY HERBS FROM THE *LAMIACEAE* FAMILY AS *IN VIVO* MODULATORS OF INSULIN SIGNALING AND IMPACT ON DISEASE

Introduction

Insulin and Insulin like signaling pathway

The insulin/insulin like signaling (INS/ILS) pathway is one of the most vital and conserved regulatory pathways (Barbieri et al., 2003). Proper function of this pathway results in a balance between nutrient metabolism, growth, or cellular defenses and longevity (K. Lin et al., 2001). Genetic, environmental or nutrient induced metabolic dysregulation of this pathway by stress has been implicated in development of chronic metabolic, mitogenic, and aging related diseases (Hotamisligil, 2006).

The INS/ILS pathway is initiated at the cell membrane when either insulin or an insulin-like growth factor (IGF) binds to the receptor (IGF-IR) (Lant & Storey, 2010). The metabolic pathway requires insulin for glucose utilization, while the IGF pathway regulates mitogenic activities (Rafalski & Brunet, 2011). When insulin or IGF binds to the receptor it activates an intrinsic tyrosine phosphorylation allowing for recruitment and interaction of insulin receptor substrates (IRS) with phosphatidylinositol 3-kinase (PI_3K) (Barbieri et al., 2003). Then PI_3K phosphorylates the membrane lipid

phosphatidylinositol 4,5-bisphosphate (PIP_2), forming phosphatidylinositol (3,4,5)trisphosphate (PIP_3) (Osaki et al., 2004) (Figure 1). PIP_3 then amplifies the signaling cascade as a second messenger activating 3'-phosphoinositide dependent kinase-1 (PDK1) which phosphorylates a serine/threonine protein kinase known as AKT or protein kinase B (PKB) (Osaki et al., 2004) (Figure 1).

Metabolically, elevated glucose in the blood stimulates insulin release which via activation of AKT facilitates glucose transporter 4 (GLUT-4) relocation to the cell membrane for glucose uptake (Kanzaki & Pessin, 2003). Insulin mediated activation of ILS also inhibits phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6pase) resulting in less free glucose in the cells (Fritsche et al., 2008). Two regulatory molecules that can repress insulin signaling by preventing activation of AKT are the direct acting protein phosphatase 2 A (PP2A) and phosphatase and tensin homologue (PTEN) which inhibits PI_3K (Shaw & Dillin, 2009) (Figure 1).

Mitogenic activity of insulin signaling is regulated by IGF and mediated via activation of AKT (van der Heijden & Bernards, 2010). AKT activates the mammalian target of rampamycin (mTOR) that transcribes eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) leading to inhibition of protein synthesis, cell growth and cell division (Mora et al., 2004). Both PDK-1 and mTOR can phosphorylate and activate serum glucocorticoid-responsive kinase 3 (SGK-3), which when active (phosphorylated) induces glycogenesis, protein synthesis, and promotes PI_3K mediated ILS (Mora et al., 2004). Furthermore, AKT and SGK-3 phosphorylate the forkhead box transcription factors (FOXO) and prevents its nuclear translocation, necessary for transcription expression of target genes coding for antioxidant enzymes such as superoxide dismutase

(SOD), catalase (CTL), DNA repair enzymes, apoptotic genes, genes that prevent cell cycling, PEPCK, and G6pase (Barthel et al., 2001; Prahlad & Morimoto, 2008) (Figure 1). FOXO can also be directly activated by sirtuin histone deacetylation enzymes independent of ILS (Lamming et al., 2004) (Figure 1). There is *in vivo* evidence that one type of sirtuin known as Sir2 was able to directly deacetylate FOXO allowing expression of genes under its regulation and increase in lifespan (Lamming et al., 2004).

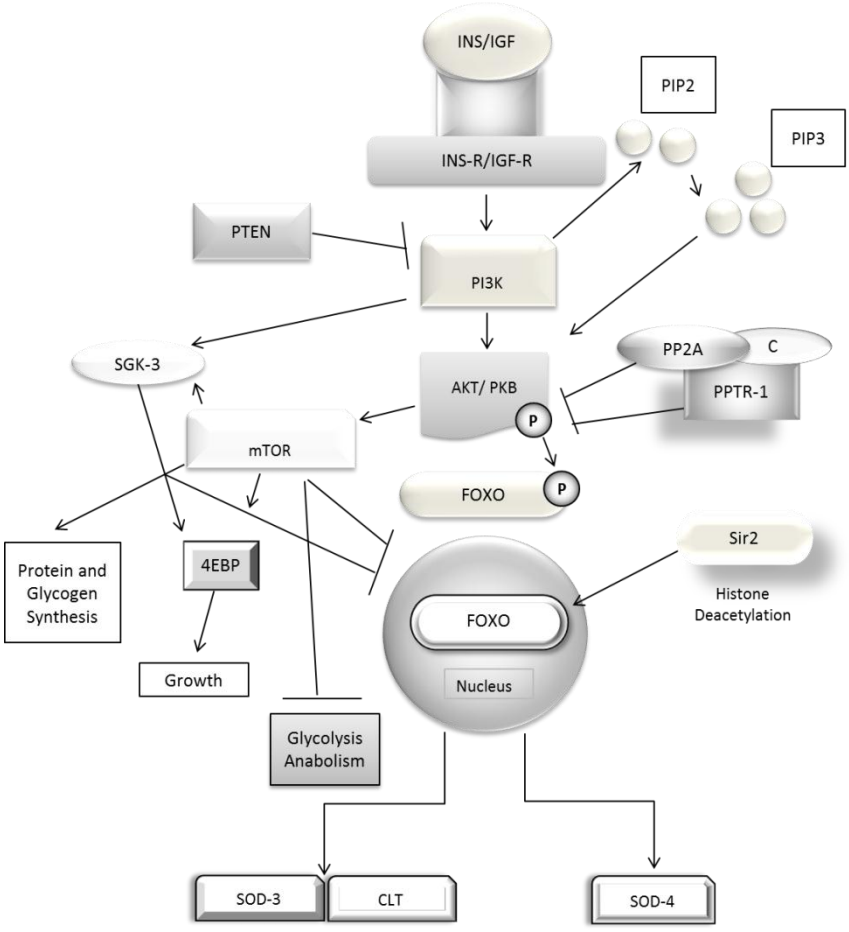

Figure 1. Insulin/ Insulin like growth factor (IGF) signaling pathway.

Dysregulation of Insulin/IGF Signaling

Diseases ranging from diabetes to various forms of cancer have been linked to targets in the insulin signaling pathway (Tao et al., 2007; Zhao & Townsend, 2009). IGF-1 related signaling is associated with cancer of the pancreas, brain, thyroid, kidney, breast, lungs, colon and leukemia (Yu & Rohan, 2000). IGF-1 via the ILS signaling and by interaction with hormones and other growth factors has mitogenic effects and has been shown to increase DNA replication, induce cell cycle progression, and inhibit apoptosis (Xue & Michels, 2007). There is evidence that inhibiting expression of IGF-1R or preventing ligand binding reduces cancer mitogenesis (Yu & Rohan, 2000). Physiologically there are binding proteins IGFBP to repress this action, but these can be degraded by proteases preventing IGFBP from transcriptional regulation (Xue & Michels, 2007). Genetic abnormalities and mutations resulting in impaired regulation or function of IGF-1, PI_3K, and PTEN have been shown to be important in the development and progression of many forms of cancer (Roy et al., 2010). PI_3K/AKT pathway and mitogen activated protein kinase (MAPK), extracellular signal-regulated protein kinase (ERK) pathway inhibition is needed to halt cancers of the pancreas (Roy et al., 2010), uterus, brain, prostate (van der Heijden & Bernards, 2010), breast, lung, colon, rectum and cervix (Ramos, 2008).

In the case of diabetes the signaling cascade is inhibited either by autoimmune destruction of pancreatic beta cells (type 1) or due to reduced sensitivity to insulin (type 2) (Klover & Mooney, 2004). Over phosphorylation and reduced numbers of IRS in hepatocytes are common markers in pre-diabetic conditions often identified as insulin-resistance (Kido et al., 2000). Over production of insulin and elevated blood lipids have

been implicated in degradation of the IRS and inhibition of the signaling cascade upstream of PI3K (Fritsche et al., 2008). Insulin resistance can also be due to altered PTEN activity causing a more unregulated inactivation of PI_3K. An additional cause of decreased insulin signaling is when IRS-1 is modified with loss of function by complexing with o-linked β-N-acetylglucosamine, known as an advanced glycation end product and a likely result of high blood glucose and lipids (Whelan et al., 2010).

Recently, neurodegeneration has been investigated as part of this pathway since insulin is needed for neuronal growth (Nishijima et al., 2010; Schubert et al., 2003). Insulin and IGF are both expressed in the brain, and can cross the blood brain barrier (Nishijima et al., 2010). *In vivo* and *in vitro* IGF-1 has been shown to stimulate neuronal stem cell growth, proliferation, and differentiation (Rafalski & Brunet, 2011). Neuronal stem cells have a greater mitogenic response to the combination of insulin and IGF than insulin alone (Rafalski & Brunet, 2011). IGF-1 has also been shown to upregulate oligodendrocytes and other growth factors for myelin generation, all of which are positively regulated by PI_3K/AKT (Chesik et al., 2008). PTEN is required for normal brain development, and reduced activity leads to enlarged brains in mice (Groszer et al., 2006). FOXO is also required to regulate neural stem cells but over proliferation due to reduced FOXO leads to cellular exhaustion and no growth of new cells (Groszer et al., 2006).

FOXO transcription of antioxidant coding genes may help limit ROS, and *in vitro* evidence shows neuronal stem cells only proliferate under a stable redox environment (Rafalski & Brunet, 2011). Impaired metabolic function can create an oxidative state in the brain, and left uncontrolled could lead to damage. Although not fully understood

neurodegerative conditions are likely due to stress, an example is the accumulation of the assumed toxic amyloid $\beta_{1\text{-}42}$ peptide seen in Alzheimer's disease (AD) which has been shown to bind to advanced glycation end products (AGE) that accumulate in type 2 diabetes (T2D) (Bierhaus & Nawroth, 2009; Maczurek et al., 2008). Furthermore, the transgenic nematode *Caenorhabditis elegans* (*C. elegans*) with decreased/mutant *daf-2* (human insulin/IGFR homologue) showed reduced amyloid β toxicity compared to wildtype animals (Dimitriadi & Hart, 2010), likely due to improved cellular defenses. The neurodegenerative Huntington's disease identified by increased glutamines (polyQ) on the Huntington protein, shows slower onset time when insulin/IGF signaling is inhibited by inactivating *age-1*/PI_3K in *C. elegans* (Dimitriadi & Hart, 2010).

Pharmacological Treatment Targets and Limitations

Cancer treatments targeting the insulin/IGF pathway include blocking IGF-1R with tyrosine kinase inhibitors and monospecific antibodies, but IGF-1R is also expressed at lower levels in non-tumor cells (Atzori et al., 2009). Small molecules have also been tested to reduce IGF-1R but these are not specific and may inhibit insulin receptors as well as IGF-1R (Atzori et al., 2009) leading to metabolic side effects. For breast cancer the drug Tamoxifen inhibits estrogen IGF-1/IGF-1R interaction, but increases the interaction in the uterus for greater risk of cancer in those cells (Nsabp et al., 2006). Whether the problem is with tyrosine kinase receptors upstream, or PTEN/PI_3K dysregulation downstream, blocking one target may possibly create undesired effects.

Effective pharmacological targets for treating diabetes would be to increase insulin production, increase insulin receptors and maintain essential kinase activity

throughout the pathway (Hui, Tang, & Go, 2009). Obese rats treated with antisense oligonucleotide (ASO) inhibitors regain PI3K/AKT signaling leading to normal glucose and insulin levels, possibly due to reductions in PEPCK (Fritsche et al., 2008). Glucagon like peptide naturally released in the fed state, has been shown to increase overall insulin response to glucose (Fritsche et al., 2008). The glucophage metformin increases insulin sensitivity without increasing insulin levels (Wright & Stanford, 2009). However, drugs of the class sulfonylureas treat diabetic symptoms by increasing insulin levels (Wright & Stanford, 2009), thereby possibly increasing the risk of tumor formation as a side effect. Other treatments such as insulin and insulin secretagogues can cause low level hypoglycemia (Miller et al., 2001), while cardiovascular complications have been seen with some thiazolidinediones (Graham et al., 2010). Prolonged use of these medications can lead to increased metabolic dysfunction and damage of many tissues (van der Heijden & Bernards, 2010).

Ideally, to prevent neurodegeneration one would allow for growth of the neurons, while simultaneously increasing the antioxidant stress response (Glass et al., 2010; Sayre et al., 2008). Resperine is a drug approved by the FDA that is reported to increase longevity, and upregulate stress response genes, in a *C. elegans* Alzheimer's disease model it increased the time for amyloid $\beta_{1\text{-}42}$ accumulation to result in paralysis (Dimitriadi & Hart, 2010). Lithium chloride and mithramycin both with FDA approval suppressed polyQ related toxicity in Huntington's disease (Dimitriadi & Hart, 2010). Despite numerous groups researching neurodegenerative diseases many of the mechanism are not fully understood therefore current treatments are not completely effective in many clinical trials.

Natural Products

Many pharmaceuticals achieve desired effects on one disease but instigate formation or aggravation of another disease as highlighted above. This results in a constant search for improved products with reduced side effects (Liebler & Guengerich, 2005; Shiri et al., 2007). Whereas, culinary herbs and spices have been commonly consumed for many years and run little risk of toxicity at dietary levels. Many effective pharmaceutical drugs are derived from plants, and generally this process involves the search for specific compounds which are isolated and synthesized (Li & Vederas, 2009). This focus on individual compounds results in no doubt effective treatments, but the loss of synergy. Synergy not only can increase treatment outcomes, but reduce side effects by containing mixtures of compounds that lower absorption. An example of this is how white willow tree bark has been used for pain relief for many years. White willow bark contains salicin, which upon consumption is metabolized to salicylic acid, the active ingredient in aspirin (Vlachojannis et al., 2011). Willow bark has been reported to be a more effective inhibitor of inflammatory cyclooxygenase (COX) II than aspirin alone, and without intestinal damage as a side effect (Vlachojannis et al., 2011). The proposed molecular mechanism involves the combination of natural compounds working in synergy. Identification of active compounds in plants is important for development of pharmaceuticals, but not when effectiveness or safety is compromised. Thus, the therapeutic value of whole foods and extracts should not be ignored.

Studies have provided evidence that diets containing whole grains, fruits, vegetables, and other plant based food are associated with decreased risks of chronic diseases (Key et al., 2004; Martinez-Gonzalez et al., 2008). Plants contain bioactive

compounds that might improve health past nutrition (Aggarwal & Shishodia, 2006; Sen et al., 2010; Shanmugam et al., 2008; Surh, 2003). Evolution has resulted in production of these bioactive chemicals as means for plants to survive climate change, nutrient shortage, and attack from other organisms (Osbourn & Lanzotti, 2009). Some major bioactive compounds in plants are phenolics, terpenoids, and alkaloids (Manach et al., 2009). Phenolic acids have many functions in plants including regulating enzyme activity, and metabolism (Robbins, 2003). Possibly due to action of these compounds herbs have been shown to act as scavengers of reactive oxygen species (ROS) *in vitro* (Dorman et al., 2003). Additionally, teas such as green tea contain several types of phenolics like epigallocatechin gallate (EGCG) which is known for anti-tumor activity (Q. Zhang et al., 2006).

A large portion of natural product consumption, especially herbs and spices is by steeping fresh and dried leaves, or ground root in hot water as tea. Spices and herbs have been used medicinally throughout recorded history as treatments for pain, antimicrobials, and easing digestive discomfort (Tapsell, 2006). Traditional medicine from around the world includes herbs as treatments for various conditions such as cancer, diabetes, and prevention of age related mental decline (Bhattacharjee, 2009; Klein et al., 2000). Recently, *in vivo* evidence suggests that herbal compounds can modify receptor or enzyme interactions (Imanshahidi & Hosseinzadeh, 2006). Since these compounds regulate such essential functions in plants it stands to reason that the same functions could possibly be modulated in higher organisms. Thus, the synergistic actions of the vast collection of phytochemicals in herbs may be successful in modulation of tightly regulated pathways such as insulin signaling.

Lamiaceae Herbs as Modulators of Insulin Signaling

Herbs have established use for more than diet, and recently medicinal history has converged with emerging scientific data especially for herbs from the family *Lamiaceae*. Based on the traditional use of these herbs for treatment of a wide variety of conditions, the therapeutic function may be to return the cellular environment to homeostasis. The vast historical reference of herbs from this family has no doubt lead to researchers identifying the possible health promoting active constituents. *Lamiaceae* herbs can contain unique compounds, but also share many in varying concentrations (S. J. Lee et al., 2005), possibly resulting in very different physiological responses. *Ocium basilium* (basil), *Origanum vulgare* (oregano), *Rosmarinus officinalis* (rosemary), *Salvia officinalis* (sage), and *Thymus vulgaris* (thyme) are commonly consumed in the human diet and are known to provide health promoting effects.

Basil contains phenolics such as caffeic acid, and rosmarinic acid which are seen in most *Lamiaceae* herbs (Park, 2011a). There are also flavonoids like luteolin, and quercetin, along with various terpenoids (Park, 2011a). Basil is known as an exceptional ROS scavenger *in vitro* (S. J. Lee et al., 2005), and in mice organ tissue it has been shown to increase levels of the stress response antioxidants glutathione reductase (GSR), reduced glutathione (GSH), SOD, and CTL (Dasgupta et al., 2004). Streptozotocin induced diabetic rats were reported to have lower blood glucose, triglycerides, cholesterol, without altered plasma insulin levels upon aqueous basil extract treatment (Zeggwagh et al., 2007). Basil and a component linalool reduced DNA mutations in a bacterial model at a similar level that had been previously achieved with the antioxidant vitamin E (Beric et al., 2008). Oxidative damage to neurons in ischemic brain cells of

mice was reduced in response to basil, and the suggested mechanism was prevention of lipid peroxidation by an increased antioxidant response evaluated by the thiobutaric acid reactive substances (TBARS) test (Bora et al., 2011).

Oregano has similar phenolics and terpenoids as basil, as well as the flavonoids apigenin, luteolin, and quercetin (Park, 2011b). Many compounds contained in oregano have had success quenching ROS *in vitro* (Yoshino & Higashi, 2006). Recently Srihari et al., (2008) reported the bioactive compounds in oregano extract improved the antioxidant status in cancer induced rat intestine. When compared to control oregano treated mice displayed an elevated stress response with increased endogenous antioxidant levels of SOD, CTL and GSH, and lower lipid peroxidation byproducts (Srihari et al., 2008). Additionally, dietarily relevant doses of oregano were effective at inducing apoptosis in a human adenocarcinoma colon cancer cell model with the suggested mechanism again being due to an upregulation in antioxidant enzymes (Savini et al., 2009). Reduction in diabetic symptoms of rats in response to aqueous oregano was displayed by normal blood glucose levels on the fourth day of once daily dosing, with no significant change in insulin levels (Lemhadri et al., 2004). Currently there is minimal information on benefits of consuming oregano extract on neurodegeneration, however with induction of antioxidant enzymes and stabilization of insulin oregano may be neuroprotective.

Rosemary is composed of various phenolics such as rosmarinic acid, the flavonoid hesperidin, the terpenoids carnosic acid, carnasol, and rosmanol (Al-Sereiti et al., 1999). Rosemary is well known for antioxidant and antimicrobial activity (Naghibi et al,, 2005). Multiple extracts and bioactive constituents of rosemary were evaluated for *in vitro* effect on human cancer cells (Yesil-Celiktas et al., 2010). These included human

small cell lung, prostate, liver, breast and myeloid leukemia, all rosemary extracts and fractionated compounds were toxic to the cell lines at low doses but carnosic acid was most effective (Yesil-Celiktas et al., 2010). Bakirel et al., reported in 2007 ethanol extracts of rosemary reduced blood glucose, and increased insulin levels of rabbits with induced diabetes. They also noted increased levels of the antioxidants SOD, and CTL in these animals (Bakirel et al., 2007).

Sage contains chlorogenic acid as well as many other phenolics and flavonoids seen in basil, oregano, and rosemary (Kintzios, 2000). Important terpenoids are cineole, thujone, hispidulin, ursolic acid, as well as beta and stigma-sterol (Kintzios, 2000). Sage is known for its *in vitro* antioxidant capacity (Matsingou et al., 2003), and has been shown to increase erythrocyte SOD and CTL levels *in vivo* (Sá et al., 2009). Slamenova et al., (2004) reported horminone and acetyl horminone diterpenoids extracted from sage induced apoptosis effectively in human hepatoma cells at low doses, as well as human colon carcinoma cells at higher concentrations. In a rodent model sage tea increased glucose uptake in the liver and decreased fasting gluconeogenesis (Lima et al., 2006). Sage is a potential treatment for improved insulin function (Lima et al., 2006), and has shown positive results in an AD clinical trial (Akhondzadeh et al., 2003). Through unexplained mechanisms emerging evidence indicates that sage is simultaneously therapeutic for cancer, diabetes, and neurodegeneration.

Thyme has a similar content of phenolics and flavonoids as do the previously discussed herbs (Fecka & Turek, 2008; Hossain et al., 2010). However, thyme differs in thymol and carvacol terpenoid content (Youdim & Deans, 2000). Thyme and its compounds have been tested for antimicrobial activity, as well as *in vitro* antioxidant

capacity (Amarowicz et al., 2009; Solomakos et al., 2008). Youdim & Deans, (2000) found that the stressful oxidative cellular environment observed in brain tissues of older rodents due to oxidation of phospholipids was reduced by increased endogenous antioxidant production upon thyme oil and thymol treatment. Additionally, it was shown that thyme oil was an effective *in vitro* treatment on three human cancer cell types with greatest toxicity on prostate carcinoma cells, followed by weaker effects on lung carcinoma and breast cancer cells (Zu et al., 2010). Although information is lacking on thyme modulation of insulin signaling relating to diabetes, positive results for other herbs in this family warrant an investigation.

Even with encouraging *in vivo* work using *Drosophila, Nematodes,* or rodents (Lima et al., 2006; Wentzell & Kretzschmar, 2010; Zhao & Townsend, 2009) the main body of research remains *in vitro* (Asadi et al., 2010; Mazzio & Soliman, 2009; Slamenova et al., 2004). Additionally, many current *in vivo* models use genetic knockouts/mutants that address only single factors of disease development (Sá et al., 2009; Srihari et al., 2008). Moreover, the dosages used *in vitro* and *in vivo* studies may be toxic, or rely on purified compounds often ignoring effect of whole extracts. As a result there is a lack of comprehensive understanding of the mechanism of action of *Lamiaceae* herbs *in vivo* on modulating critical pathways in eukaryotic systems such as the ILS. The objective was to evaluate the *in vivo* dose-dependent effect of *Lamiaceae* herbs on modulation of stress response signaling in the ILS pathway, and the implications of modulation on prevention or management of chronic and neurodegenerative disease in *C. elegans* models.

Methods

Nematode Propagation and Treatment

The *C. elegans* is a microscopic nematode with a completely mapped genome. This model organism provides numerous benefits including translucence for uninhibited evaluation of green fluorescent protein (GFP) expression, and a vast array of available transgenic strains suitable for screening of entire signaling pathways. Additionally, *C. elegans* are a relatively inexpensive *in vivo* model that provides large amounts of data in a short period of time. Transgenic strains of *C. elegans* with GFP promoter constructs of genes relevant to ILS pathway (Table 1) 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 MgSO4, 5mg/mL Cholesterol in ethanol, 1M KPO4) at 18-20^{o}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 50µl of *Escherichia coli* OP50 overnight cultures and incubated for 9 hours at 37^{o}C. Plates containing a transgenic worm expressing the gene of interest propagated on NGM with only *E. coli* OP50 served as a control for that strain. The strains of *C. elegans* were maintained by picking 2 adult worms onto freshly inoculated NGM plates every 4-7 days.

Treatment plates contained aqueous extracts of *Lamiaceae* herbs at concentrations of 1 mg/ml, 5 mg/ml, and 10 mg/ml. Aqueous extracts of *Lamiaceae* herbs were prepared by heating 1.5g of the herb in 30mL of distilled water at 60^{o}C for 30 minutes. The mixture was vacuum-filtered through Whatman filter paper. Then the extract was

sterilized by 0.2µm syringe filters to avoid microbial contamination. Extracts of *Lamiaceae* herbs were added to the NGM media just prior to pouring NGM agar plates (Caldicott et al., 1994). *E. coli* OP50 was mixed with the extracts in the same test concentrations as the media, and treatment plates were inoculated as described above. One mature adult worm was then transferred to treatment plates (5 plates per concentration) and allowed to lay eggs, hatch and grow to the L4/young adult stage.

Fluorescence Imaging and Quantification

Images of 1 or 2 (L4/young adult) worms from each plate were captured using the Nikon SMZ1500 fluorescence microscope with Ri1/Qi1 CCD camera. Prior to capturing the images, the worms were temporarily immobilized by chilling the plates on ice for 15 minutes. The relative fluorescence with respect to control (NGM with *E. coli* OP50) was then quantified using the National Institute of Health's ImageJ software (Iser & Wolkow, 2007).

Statistical Analysis

Fold change in relative fluorescence (RF) was calculated between controls and each treatment concentration. Statistical analyses of data were performed using a one-tailed t-test, since we expected to see either an increase or decrease in expression compared to control. A p value ≤ 0.05 was considered statistically significant. A maximum of 10 and minimum of 8 images were collected for every treatment.

Table 1. List of *C. elegans* genes evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Strain	Gene	Human Homolog	Wormbase-Gene ID
GR1352	***daf-16a***	Forkhead Box O (FOXO)	WBGene00000912
TJ356	***daf 16***	Forkhead Box O (FOXO)	WBGene00000912
BC14074	***daf-2***	Insulin/Insulin growth factor (IGF) receptor	WBGene00000898
BC10837	***age-1***	Phosphoinositide 3-kinase (PI_3K)	WBGene00000090
BC10950	***let-92***	Protein Phosphatase 2A	WBGene00002363
BC14613	***pptr-1***	Protein Phosphatase 2A regulatory subunit	WBGene00012348
CF1553	***sod-3***	Iron/Manganese superoxide dismutase	WBGene00004932
BC13632	***sod-4***	Copper/Zinc superoxide dismutase	WBGene00004933
GA800	***ctl***	Catalase	WBGene00000831
UL3294	***sir2.1***	Sirtuin 2	WBGene00004800
UL3351	***sir2.1***	Sirtuin 2	WBGene00004800
CF2266	***ins-7***	Insulin/Insulin growth factor (IGF)-1-like peptide	WBGene00002090
GR1455	***daf-28***	Insulin-4 (ins-4), Human insulin	WBGene00000920
BL5752	***ida-1***	Type I diabetes autoantigens IA-2/phogrin	WBGene00002048

Results

Effect of Basil on Insulin Signaling

The gene expression of *daf-2* (BC14074) was evaluated at three concentrations of basil (1 mg /ml, 5 mg/ml and 10 mg/ml) (Table 2). When compared to control an overall pattern of decreased expression was observed. However, significant decreases in expression were achieved only in response to 5 mg/ml (RF=0.70, $p=<0.05$), and the 10 mg/ml (RF=0.72, $p=0.001$) concentrations (Table 2). The effect of basil on the gene *age-1* (BC10837) was similar to *daf-2*, with a significant decrease in *age-1* expression observed only at 5 mg/ml (RF=0.77, $p=<0.05$) and 10 mg/ml (RF=0.74, $p=<0.05$)

concentrations (Table 2). When compared to the control expression, *let-92* (BC10950) was significantly upregulated at 5 mg/ml (RF=1.52, *p=0.008*) while the lower (1 mg/ml) concentration resulted in reduced expression (RF=0.85, *p=0.023*). The effects of basil on expression of *pptr-1* (BC14613), the regulatory subunit of *let-92* were non-dose dependent. At the lowest concentration (1 mg/ml) there was a significant decrease in expression (RF=0.90, *p=0.005*), while increased concentrations of 5 mg/ml (RF=1.13, *p=<0.05*), and 10 mg/ml (RF=1.04, *p=<0.05*) resulted in significantly higher expression over control (Table 2). *Daf-16*, a downstream transcription factor for many endogenous antioxidants was tested in two *C. elegans* strains. Isoform *daf-16α* (GR1352) expression was similar at both 1 mg/ml (RF=1.80, *p=<0.05*) and 5 mg/ml (RF=1.80, *p=<0.05*), at10 mg/ml of basil was 1.53 fold (*p=<0.05)* higher than control *daf-16α* expression. However, when compared to control all three concentrations significantly increased expression (Table 2). When expression of *daf-16* (TJ356) in response to basil was evaluated the results were opposite, the treatment with basil significantly decreased expression compared to control. As the concentration of basil increased from 1 mg/ml (RF=0.24, *p=<0.05*), to 5 mg/ml (RF=0.21, *p=<0.05*), to10 mg/ml (RF=0.15, *p=<0.05*) the expression of *daf-16* decreased correspondingly. Transcription factor *daf-16* regulates several genes downs stream including *sod-3* that codes for the mitochondrial iron/manganese superoxide dismutase. The expression of *sod-3* (CF1553) was significantly increased in response to basil at only the 1 mg/ml (RF=1.59, *p=0.004*) and 5 mg/ml (RF=1.42, *p=0.019)* concentrations relative to control (Table 2). Another gene *sod-4*, (BC13632) codes for the cytoplasmic antioxidant copper/zinc superoxide dismutase, and its expression was significantly upregulated at the higher two

concentrations of basil, 5 mg/ml (RF=1.23, *p=<0.05*) and 10 mg/ml (1.25 fold, p=<0.05). Expression of *ctl* (GA800) was decreased in a dose dependent manner with all basil treatments. Overall increases in basil concentration from 1 mg/ml (RF=0.91, *p=<0.05*), to 5 mg/ml (RF=0.84, *p=<0.05*) to 10 mg/ml (RF=0.81, *p=<0.05*) resulted a consistent decrease in expression relative to the control. A non-dose dependent response to basil was observed with the gene *ida-1* (BL5752), gene expression was significantly reduced at 1 mg/ml (RF=0.83, *p=0.001*), 5 mg/ml (RF=0.87, *p=0.002*), and 10 mg/ml (RF=0.82, *p=<0.05*) concentrations relative to control. In response to basil, the expression of *ins-7* (CF2266), which is downregulated regulated by active *daf-16* was similar to *ida-1*. At 1 mg/ml (RF=0.99, *p=<0.05*), 5 mg/ml (RF=0.93, *p=0.001*), and 10 mg/ml RF=0.98, *p=<0.05*) concentrations of basil resulted in non-dose dependent downregulation in gene expression. Concentrations of 1mg/ml (RF=1.47, *p=<0.05*), 5 mg/ml (RF=1.21, *p=0.001*), and 10mg/ml (RF=1.72, *p=<0.05*) basil increased expression of *daf-28* (GR1455) compared to control. Even though the response did not change linearly with increase in concentration, the expression of *daf-28* was significantly higher than the control at all doses (Table 2). The expression of *sir2.1* in response to basil treatment was evaluated in two *C. elegans* strains, and results were inconclusive. Relative to control there was a significant dose-dependent reduction in *sir2.1* expression in one strain (UL3294) at 1 mg/ml (RF=0.53, *p=<0.05*), 5 mg/ml (RF=0.52, *p=<0.05*), and 10 mg/ml (RF=0.49, *p=0.002*). In the second strain (UL3351), there was only significant effects of basil on expression of *sir2.1* at 1 mg/ml (RF=1.20, *p=0.006*), and 5 mg/ml (RF=1.08, *p=<0.05*) basil concentrations, where an increase in expression was noted relative to control (Table 2).

Table 2. Dose dependent effect of basil treatments on fold-change in gene expression relative to control.

GENE	Basil Concentration								
	1 mg/ml			5 mg/ml			10 mg/ml		
	RF	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
daf-16a	1.800	0.069	***<0.05***	1.801	0.073	***<0.05***	1.530	0.054	***<0.05***
daf-16	0.237	0.034	***<0.05***	0.212	0.007	***<0.05***	0.153	0.007	***<0.05***
daf-2	0.900	0.036	*0.074*	0.704	0.060	***<0.05***	0.718	0.044	***0.001***
age-1	0.952	0.041	*0.231*	0.766	0.029	***<0.05***	0.742	0.027	***<0.05***
let-92	0.850	0.097	***0.023***	1.520	0.275	***0.008***	0.920	0.085	*0.108*
pptr-1	0.900	0.464	***0.005***	1.130	0.586	***<0.05***	1.040	0.523	***<0.05***
sod-3	1.590	0.193	***0.004***	1.420	0.067	***0.019***	0.990	0.042	*0.372*
sod-4	1.013	0.053	*0.155*	1.234	0.044	***<0.05***	1.247	0.043	***<0.05***
ctl	0.910	0.074	***<0.05***	0.840	0.037	***<0.05***	0.810	0.048	***<0.05***
sir2.1	0.530	0.527	***<0.05***	0.520	0.516	***<0.05***	0.490	0.490	***0.002***
sir2.1	1.197	0.220	**0.006**	1.078	0.199	***<0.05***	1.269	0.216	0.063
ins-7	0.990	0.052	***<0.05***	0.932	0.042	**0.001**	0.978	0.052	***<0.05***
daf-28	1.466	0.110	***<0.05***	1.211	0.072	**0.001**	1.725	0.157	***<0.05***
ida-1	0.832	0.036	**0.001**	0.869	0.032	**0.002**	0.825	0.029	***<0.05***

Basil was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=6-8.

Effect of Oregano on Insulin Signaling

Daf-2 expression was significantly increased at 1 mg/ml (RF=1.13, *p=<0.05*), 5 mg/ml (RF=1.23 fold, *p=<0.05*), and 10 mg/ml (RF=1.18 fold, *p=<0.05*) concentrations of oregano relative to control (Table 3). *Age-1* expression was significantly downregulated from control at only 5 mg/ml (RF=0.84, *p=0.007*), 1 mg/ml and 10 mg/ml concentrations did not impact expression (Table 3). In response to oregano *pptr-*1expression was significantly increased at all three concentrations. The highest response was seen at 5 mg/ml (RF=1.58, *p=<0.05*), followed by 10 mg/ml (RF=1.41, *p=<0.05*) and 1 mg/ml (RF=1.32, *p=<0.05*) (Table 3). A significant multi-fold increase in *let-92* expression occurred at all three concentrations of oregano. Relative to control at 1 mg/ml

a 5.10 fold increase (*p=<0.05*) was observed. At 5 mg/ml and 10 mg/ml the expression of *let-92* was 3.18 fold (*p=<0.05*) and 4.36 fold (*p=<0.05*) respectively (Table 3) The *daf-16α* isoform showed a significant increase in expression at 1 mg/ml, 5 mg/ml, 10 mg/ml concentrations [(RF=1.41, *p=0.001*), (RF=1.21, *p=0.002*), and (RF=1.36, *p=<0.05*) respectively] (Table 3). Expression of *daf-16* was also upregulated in response to oregano. Relative to control the highest increase was seen at 5 mg/ml (RF=1.86, *p=<0.05*), followed by 10 mg/ml RF=1.68, *p=<0.05*), and 1 mg/ml (RF=1.59, *p=<0.05*) (Table 3). Expression of *sod-3* in response to oregano increased over control at all three concentrations (Table 3). At 1 mg/ml the expression increased 2.07 fold (*p=0.001*), followed by 1.41 fold at 5 mg/ml (*p=0.033*), and 1.30 fold at 10 mg/ml, (*p=0.040*) (Table 3). The gene *sod-4* was significantly downregulated at 1 mg/ml (RF=0.66, *p=<0.05*), 5 mg/ml (RF=0.79 fold, *p=<0.05*), and 10 mg/ml (RF=0.80, *p=<0.05*) (Table 3). Treatment with oregano significantly decreased expression of *ctl* at1 mg/ml (RF=0.79, *p=<0.05),* 5 mg/ml (RF=0.94, *p=<0.05*), and 10 mg/ml (RF=0.98, *p=<0.05*) relative to control (Table 3). The expression of *ida-1*was significantly reduced at 1mg/ml (RF= 0.89, *p=0.020*), but expression at 10 mg/ml (RF=1.02 fold, *p=0.010*) was increased (Table 3). The gene for *ins-7* was significantly downregulated at 1 mg/ml (RF=0.965, *p=<0.05*), but was upregulated at 5 mg/ml (RF=1.05, *p=0.021*), and 10 mg/ml (RF=1.12, *p=<0.05*) (Table 3). *Daf-28* expression was not significantly different from control at 1mg/ml, but was significantly lower at the 5 mg/ml (RF=0.79, *p=0.008*) and 10 mg/ml (RF=0.73, *p=0.008*) concentrations (Table 3). The gene expression of *sir2.1* (UL3294) in response to oregano resulted in significant decreases at all concentrations [1 mg/ml (RF=0.47, *p=<0.05*), 5 mg/ml (RF=0.46, *p=<0.05*) and 10 mg/ml (RF=0.46, *p=<0.05*)] when

compared to control (Table 3). The expression of the *sir2.1* in the second strain (UL3351) did not significantly change in response to oregano at 1 mg/ml, there was however significant decrease in expression at 5 mg/ml (RF=0.93, *p=0.038*), and a significant increase at 10 mg/ml (RF=1.13, *p=0.015*) relative to control (Table 3).

Table 3. Dose dependent effect of oregano treatments on fold-change in gene expression relative to control.

	Oregano Concentration								
	1 mg/ml			**5 mg/ml**			**10 mg/ml**		
GENE	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
daf-16a	1.407	0.137	***0.001***	1.213	0.061	***0.002***	1.362	0.042	***<0.05***
daf-16	1.591	0.128	***<0.05***	1.863	0.091	***<0.05***	1.676	0.124	***<0.05***
daf-2	1.130	0.043	***<0.05***	1.230	0.074	***<0.05***	1.180	0.047	***<0.05***
age-1	0.995	0.041	*0.136*	0.847	0.060	***0.007***	0.973	0.039	*0.463*
let-92	5.100	0.682	***<0.05***	3.180	0.325	***<0.05***	4.360	0.527	***<0.05***
pptr-1	1.320	0.663	***<0.05***	1.580	0.789	***<0.05***	1.410	0.702	***<0.05***
sod-3	2.070	0.327	***0.001***	1.410	0.036	***0.033***	1.300	0.096	***0.040***
sod-4	0.663	0.071	***<0.05***	0.790	0.063	***<0.05***	0.804	0.045	***<0.05***
ctl	0.790	0.091	***<0.05***	0.940	0.041	***<0.05***	0.980	0.026	***<0.05***
sir2.1	0.470	0.027	***<0.05***	0.460	0.016	***<0.05***	0.460	0.020	***<0.05***
sir2.1	0.957	0.074	0.191	0.933	0.062	**0.038**	1.127	0.076	***0.015***
ins-7	0.965	0.041	***<0.05***	1.049	0.027	**0.021**	1.122	0.172	***<0.05***
daf-28	0.952	0.090	0.216	0.789	0.101	**0.008**	0.731	0.110	***<0.05***
ida-1	0.889	0.020	***<0.05***	0.926	0.051	0.293	1.017	0.137	***0.010***

Oregano was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Effect of Rosemary on Insulin Signaling

Relative to control, treatment with rosemary significantly increased expression of *daf-2* at all concentrations. *Daf-2* expression at 1 mg/ml was (RF=1.34, *p=<0.05*), 5 mg/ml (RF=1.08, *p=<0.05*) and 10 mg/ml (RF=1.01, *p=<0.05*) was significantly higher than control (Table 4). A dose dependent decrease in *age-1* expression was observed with treatments relative to control, at 1 mg/ml, 5 mg/ml, and 10 mg/ml the fold decreases in

expression were 0.71, (*p=<0.05*), 0.76, (*p=0.001*) and 0.79 (*p=0.010*) respectively (Table 4). When compared to control *let-92* expression was not different from control at 5 mg/ml. However a significant downregulation was observed at 1 mg/ml (RF=0.82, *p=0.017*) and 10 mg/ml (RF=0.63, *p=0.001*) (Table 4). Rosemary treatment at any concentration did not result in a significant response by *pptr-1* (Table 4). *Daf-16α* expression was significantly increased at 1 mg/ml (RF=1.39, *p=0.002*), 5 mg/ml (RF=1.35, *p=0.001*), and 10 mg/ml (RF=1.29, *p=0.001*) (Table 4). *Daf-16* expression in response to rosemary resulted in a 2.13 fold (*p=<0.05*) increase at 10 mg/ml, followed by 2.11 fold (*p=<0.05*) at 5mg/ml, and 1.97 fold (*p=<0.05*) at 1 mg/ml (Table 4). *Sod-3,* which codes for copper/zinc superoxide dismutase showed significant increases in expression at all concentrations of rosemary [1 mg/ml (RF=1.68, *p= 0.016),* 5 mg/ml (RF=1.80, *p=<0.05*), and 10 mg/ml (RF=1.68, *p=<0.05)*] relative to control (Table 4). Treatment with rosemary extract resulted in significant downregulation of *sod-4* expression at all concentrations compared to control (Table 4). Similar reductions in expression relative to control occurred at 1 mg/ml (RF=0.88, *p=<0.05*), and 5 mg/ml (RF=0.89, *p=<0.05*), while the highest downregulation was observed at 10 mg/ml (RF=0.75, *p=<0.05*) (Table 4). Expression of c*tl* in response to rosemary was significantly reduced at 1 mg/ml (RF=0.93, *p=<0.05*), and 5 mg/ml (RF=0.92, *p=<0.05*). However, a significant increase in *ctl* expression was observed at 10 mg/ml (RF=1.03 fold, *p=<0.05*) when compared to control (Table 4). Significant changes in *ida-1* expression were seen at all three test concentrations of rosemary. At the lowest concentration [1 mg/ml (RF=1.04, *p=0.040*)] an increase in expression occurred, but significant downregulation was seen at 5mg/ml (RF=0.82, *p=<0.05*), and 10 mg/ml

(RF=0.85, *p=<0.05*) (Table 4). *Ins-7* expression in response to rosemary was increased at 1 mg/ml (RF=1.07 fold, *p=0.044*), 5 mg/ml (RF=1.02, *p=0.044*), and 10 mg/ml (RF=1.03, *p=<0.05*) all at similar levels (Table 4). In response to rosemary *daf-28* was upregulated 1.65 fold (*p=<0.05*), 1.64 fold (*p=<0.05*), and 1.37 fold (*p=<0.05*) over control at 1 mg/ml, 10 mg/ml, and 5 mg/ml respectively (Table 4). A similar level of downregulation relative to control was seen at all concentrations for one of the *sir2.1* (UL3294) strains [1 mg/ml (RF=0.29, *p=<0.05*), 5 mg/ml (RF=0.29 fold, *p=0.002*), and 10 mg/ml (RF=0.24, *p=000*)] (Table 4). Expression of the second *sir2.1* (UL3351) strain also showed reduced expression in response to rosemary at 1 mg/ml (RF=0.80, *p=<0.05*), 5 mg/ml (RF=0.82, *p=<0.05*), and 10 mg/ml (RF=0.77, *p=<0.05*) when compared to control (Table 4).

Table 4. Dose dependent effect of rosemary treatments on fold-change in gene expression relative to control.

	Rosemary Concentration								
	1 mg/ml			**5 mg/ml**			**10 mg/ml**		
GENE	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
daf-16a	1.329	0.128	***0.002***	1.349	0.099	***0.001***	1.292	0.044	***0.001***
daf-16	2.114	0.057	***<0.05***	1.966	0.097	***<0.05***	2.131	0.074	***<0.05***
daf-2	1.341	0.093	***<0.05***	1.083	0.050	***<0.05***	1.097	0.033	***0.001***
age-1	0.710	0.028	***<0.05***	0.764	0.071	***0.001***	0.793	0.091	***0.010***
let-92	0.820	0.061	***0.017***	0.920	0.237	***0.389***	0.630	0.071	***0.001***
pptr-1	0.760	0.091	*0.483*	0.810	0.093	***0.075***	0.750	0.081	*0.331*
sod-3	1.680	0.388	***0.016***	1.800	0.189	***<0.05***	1.680	0.242	***<0.05***
sod-4	0.877	0.059	***<0.05***	0.897	0.043	***<0.05***	0.745	0.026	***<0.05***
ctl	0.930	0.035	***<0.05***	0.920	0.040	***<0.05***	1.030	0.029	***<0.05***
sir2.1	0.290	0.030	***<0.05***	0.290	0.034	***0.002***	0.240	0.053	***<0.05***
sir2.1	0.797	0.049	***<0.05***	0.822	0.034	***<0.05***	0.774	0.043	***<0.05***
ins-7	1.071	0.049	***0.044***	1.023	0.039	***0.006***	1.029	0.065	***<0.05***
daf-28	1.652	0.149	***<0.05***	1.370	0.064	***<0.05***	1.644	0.120	***<0.05***
ida-1	1.043	0.057	***0.040***	0.820	0.026	***<0.05***	0.850	0.027	***<0.05***

Rosemary was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Effect of Sage on Insulin Signaling

Daf-2 expression, when tested with sage was significantly increased at 1 mg/ml (1.45 fold, $p=<0.05$), 5 mg/ml (1.42 fold, $p=<0.05$), and 10 mg/ml (1.58 fold, $p=0.003$) relative to control (Table 5). *Age-1* expression in response to sage was downregulated only at 5 mg/ml (RF=0.86, $p=<0.05$), and 10 mg/ml (RF=0.92, $p=0.001$), no difference from control was distinguished at 1mg/ml (Table 5). There was increased expression of *let-92* in response to sage across all test concentrations [1 mg/ml (RF=3.84, $p=<0.05$), 5 mg/ml (RF= 3.37, $p=0.004$), and 10 mg/ml (RF=3.78, $p=<0.05$)] compared to control expression (Table 5). The regulatory subunit *pptr-1* was increased significantly at 1 mg/ml (RF=1.26, $p=<0.05$), and 10 mg/ml (RF=1.17 fold, $p=<0.05$), but 5 mg/ml of sage did not result in significant change over control (Table 5). Expression of *daf-16α* in response to sage significantly increased at all concentrations (Table 5). When compared to control, the expression was highest at 5 mg/ml (RF=1.35, $p=0.001$), followed by 10 mg/ml (1.33, $p=0.001$) and then 1 mg/ml (1.13 fold, $p=0.001$) (Table 5). *Daf-16* expression was upregulated at 1 mg/ml (RF=1.60, $p=0.008$), 5mg/ml (RF=1.58, $p=<0.05$), and 10 mg/ml (RF=1.42, $p=<0.05$) of sage relative to control (Table 5). Treatment with sage did not change expression of *sod-3* compared to control at 1 mg/ml, 5 mg/ml, or 10 mg/ml (Table 5). A significant downregulation in *sod-4* was observed at 5 mg/ml (RF=0.64, $p=<0.05$) and 10 mg/ml (RF=0.62, $p=<0.05$), yet there was no difference from control at 1 mg/ml (Table 5). Gene expression of *ctl* in response to sage was elevated at all three test concentrations in a dose-dependent manner (Table 5). The highest increase in expression was at 1mg/ml (RF=1.13, $p=<0.05$) followed by 5mg/ml (RF=1.05, $p=<0.05$) and then 10 mg/ml (RF=1.03, $p=<0.05$) (Table 5). Compared to

control, gene expression of *ida-1* was reduced at 1 mg/ml (RF=0.88, *p=0.007*), 5 mg/ml (RF=0.80, *p=<0.05*), and 10 mg/ml (RF=0.89, *p=0.004*) when treated with sage (Table 5). Expression of *ins-7* was significantly reduced at 1 mg/ml (RF=0.99, *p=0.001*), upregulated at 10 mg/ml (RF=1.38, *p=<0.05*), and no different from control at 5 mg/ml (Table 5). The expression of *daf-28* was significantly upregulated by 1.21 fold (*p=0.033*), 1.17 fold (*p=<0.05*) and 1.21 fold (*p=<0.05*) relative to control in response to sage at 1 mg/ml, 5 mg/ml, and 10mg/ml concentrations respectively (Table 5). *Sir2.1* (UL3294) treatment with sage resulted in multi-fold upregulations over control at 1 mg/ml (RF=4.23, *p=<0.05*), 5 mg/ml (RF=3.77, *p=0.001*), and 10 mg/ml (RF=4.29, *p=<0.05*) (Table 5). In response to sage, the second *sir2*.1 (UL3351) strain was upregulated at 1mg/ml (RF=1.10, *p=0.009*) and 5 mg/ml (RF=1.13, *p=0.029*), but a significant downregulation from control expression was observed at 10 mg/ml (RF=0.85, *p=<0.05*) (Table 5).

Table 5. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

	Sage Concentration								
	1 mg/ml			**5 mg/ml**			**10 mg/ml**		
GENE	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
daf-16a	1.133	0.080	***0.001***	1.351	0.042	***0.001***	1.330	0.030	***0.001***
daf-16	1.596	0.248	***0.008***	1.584	0.101	***<0.05***	1.424	0.136	***<0.05***
daf-2	1.450	0.103	***<0.05***	1.418	0.051	***<0.05***	1.580	0.179	***0.003***
age-1	0.953	0.036	*0.139*	0.856	0.038	***<0.05***	0.920	0.028	***0.001***
let-92	3.840	0.520	***<0.05***	3.370	0.669	***0.004***	3.780	0.278	***<0.05***
pptr-1	1.260	0.158	***0.002***	0.970	0.085	***0.001***	1.170	0.047	***<0.05***
sod-3	0.770	0.020	*0.090*	0.780	0.020	*0.100*	0.830	0.087	*0.108*
sod-4	1.045	0.077	*0.092*	0.640	0.036	***<0.05***	0.615	0.035	***<0.05***
ctl	1.130	0.043	***<0.05***	1.050	0.031	***<0.05***	1.030	0.038	***<0.05***
sir2.1	4.290	0.581	***<0.05***	3.770	0.748	***0.001***	4.230	0.311	***<0.05***
sir2.1	1.105	0.063	***0.009***	1.133	0.089	***0.029***	0.852	0.031	***<0.05***
ins-7	0.990	0.046	***0.001***	1.170	0.038	*0.458*	1.381	0.062	***<0.05***
daf-28	1.214	0.130	***0.033***	1.169	0.051	***<0.05***	1.209	0.056	***<0.05***
ida-1	0.879	0.470	***0.007***	0.799	0.423	***<0.05***	0.893	0.471	***0.004***

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Effect of Thyme on Insulin Signaling

Expression of the insulin receptor *daf-2* in response to thyme was significantly reduced from control 0.74 fold (*p=0.002)* at 1 mg/ml and 0.67 fold at both 5mg/ml and 10mg/ml (*p=<0.05, p=<0.05* respectively) (Table 6). Thyme exposure resulted in downregulation of *age-1*only at concentrations of 5 mg/ml (RF=0.86, *p=<0.05*), and 10 mg/ml (RF=77.0, *p=<0.05*), 1 mg/ml did not differ from control (Table 6). *Let-92* expression at 1 mg/ml (RF=0.65, *p=<0.05*), and 5 mg/ml (RF=0.75, *p=0.015*) was significantly reduced, at 10 mg/ml of thyme significant response over control was not observed (Table 6). Response to thyme resulted in significant downregulation of *pptr-1* only at 5 mg/ml (RF=0.87, *p=<0.05*) relative to control, notable changes were not

observed in expression at 1 mg/ml or 10 mg/ml (Table 6). The response in *daf-16a* expression seen upon treatment with thyme resulted in significantly increased expression at all three concentrations (Table 6). The highest increase in expression from control was observed at 1 mg/ml (RF=1.65, *p=<0.05*), followed by 5 mg/ml (RF=1.27, *p=<0.05*), and 10 mg/ml (RF=1.28, *p=<0.05*) (Table 6). *Daf-16* expression upregulated multi-fold in response to thyme with changes of 2.98 fold (*p=<0.05*) at 5 mg/ml, 2.49 fold (p=<0.05) at 1 mg/ml, and 2.42 fold (*p=0.001*), at 10 mg/ml (Table 6). Expression of *sod-3* was increased in response to thyme significantly only at 1 mg/ml (RF=1.37, *p=0.036*), and 5 mg/ml (RF=1.44, *p=0.005*), no difference was seen at 10 mg/ml when compared to control (Table 6). Expression of *ctl* significantly increased in response to thyme at all three concentrations [1 mg/ml (RF=1.40, *p=<0.05*), 5 mg/ml (RF=1.49, p=<0.05), and 10 mg/ml (RF=1.41, p=<0.05)] (Table 6). Downregulation of *sod-4* expression was observed in response to treatment with each concentration of thyme relative to control. This decreased expression was dose-dependent with fold changes of 0.87 (p=<0.05), 0.81 (*p=<0.05*), and 0.78 (*p=<0.05)* relative to control at 1 mg/ml, 5 mg/ml and 10 mg/ml respectively (Table 6). *Ida-1* expression was only significantly downregulated at 10 mg/ml (RF=0.83, *p=<0.05*), 1 mg/ml and 5 mg/ml did not differ from control (Table 6). Significant reductions in *ins-7* expression were seen at the 1 mg/ml (RF=0.89, *p=0.004*), 5 mg/ml (RF=0.97, *p=0.030*), 10 mg/ml (RF=0.86, *p=<0.05*) (Table 6). *Daf-28* gene expression in response to thyme was significantly reduced at 5 mg/ml (RF=0.48, *p=<0.05*), followed by 1 mg/ml (RF=0.61, *p=<0.05*), and 10 mg/ml (RF=0.78, *p=<0.05*) when compared to control (Table 6). Expression of *sir2.1* (UL3294) was significantly downregulated at the two lower concentrations of 1 mg/ml

(RF=0.72, *p=<0.05*), and 5 mg/ml (RF=0.83, *p=<0.05*) (Table 6). When the concentration of thyme was increased to 10 mg/ml an upregulation (RF=1.09, *p=<0.05*) of *sir2.1* (UL3294) over control was observed (Table 6). Thyme treatment in the second strain expressing *sir2.1* (UL3351) compared to control resulted in decreased expression at 1 mg/ml (RF=0.89, *p=<0.05*), 5 mg/ml (RF=0.75, *p=<0.05*), and 10 mg (RF=0.83, *p=<0.05*) concentrations (Table 6).

Table 6. Dose dependent effect of thyme treatments on fold-change in gene expression relative to control.

	Thyme Concentration								
	1 mg/ml			**5 mg/ml**			**10 mg/ml**		
GENE	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
daf-16a	1.651	0.128	***<0.05***	1.272	0.051	***<0.05***	1.279	0.098	***<0.05***
daf-16	2.420	0.349	***0.001***	2.979	0.146	***<0.05***	2.494	0.256	***<0.05***
daf-2	0.736	0.031	***0.002***	0.670	0.052	***<0.05***	0.670	0.058	***<0.05***
age-1	0.973	0.029	*0.423*	0.863	0.024	***<0.05***	0.775	0.021	***<0.05***
let-92	0.650	0.076	***<0.05***	0.750	0.047	***0.015***	0.980	0.098	*0.222*
pptr-1	0.840	-	*0.091*	0.870	-	***<0.05***	0.800	-	*0.313*
sod-3	1.370	0.052	***0.036***	1.440	0.145	***0.005***	1.210	0.011	*0.199*
sod-4	0.873	0.026	***<0.05***	0.806	0.030	***<0.05***	0.785	0.019	***<0.05***
ctl	1.400	0.036	***<0.05***	1.490	0.053	***<0.05***	1.410	0.087	***<0.05***
sir2.1	0.720	0.084	***<0.05***	0.830	0.053	***<0.05***	1.090	0.109	***<0.05***
sir2.1	0.886	0.042	***<0.05***	0.750	0.027	***<0.05***	0.831	0.030	***<0.05***
ins-7	0.890	0.043	***0.004***	0.967	0.055	***0.030***	0.863	0.030	***<0.05***
daf-28	0.614	0.054	***<0.05***	0.483	0.039	***<0.05***	0.782	0.036	***<0.05***
ida-1	0.899	0.043	*0.079*	0.914	0.058	*0.243*	0.833	0.019	***<0.05***

Thyme was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

Insulin Signaling in Response to Basil

The homologue for the transmembrane insulin/IGF receptor is *daf-2* in *C. elegans* (Prahlad & Morimoto, 2008). Reduction in expression and activation of this receptor

would decrease the propagation of the phosphorylation cascade, resulting in slower growth and metabolism but increased cellular defenses. In response to basil, expression of *daf-2* was downregulated. The available receptors, upon ligand binding activate an intrinsic tyrosine kinase. The phosphorylation of the IR allows for the recruitment of IRS and facilitates the interaction with PI_3K (*age*-1 in *C. elegans*) and propagates the signal (Lant & Storey, 2010). In our results, in addition to *daf-2*, the expression of *age-1* was also downregulated in response to basil treatment, suggesting a probable reduction in insulin/IGF signal activation. Upon interaction with *daf-2*, *age-1*/PI_3 Kinase phosphorylates PIP_2 into the second messenger PIP_3which binds and activates another kinase PDK1 (Osaki et al., 2004). Among the many cytoplasmic protein targets of activated PDK1, AKT/protein kinase B (PKB) is also phosphorylated and activated by PDK1 (Barbieri et al., 2003). Phosphorylation of the transcription factor FOXO (*daf-16* in *C. elegans*) by AKT/PKB causes cytosolic retention of *daf-16* (Kwon et al., 2010). As nuclear translocation of *daf-16* is required for transcriptional activation of several genes in antioxidant response and macronutrient metabolism, the activity of AKT/PKB is a critical important regulatory event in the modulation of several physiological processes in the cell such as growth, reproduction, apoptosis metabolism and stress response (Barbieri et al., 2003). We observed a dichotomous effect on *daf-16* expression in response to treatment with basil. However, the expression of *sod-3* (copper/manganese superoxide dismutase) which is positively regulated by *daf-16* (Hoogewijs et al., 2008) was significantly upregulated in response to basil treatment, suggesting that *daf-16* transcript (protein) was dephosphorylated and undergoing active nuclear translocation. The gene *ctl* that codes for three isoforms of the antioxidant enzyme catalase (Henderson et al., 2006)

was not different from control upon treatment with basil. Like SOD, the gene *ctl* is also under positive transcriptional regulation of *daf-16,* but its transcription is also actively regulated by redox stress and other metabolic processes (Henderson et al., 2006). It has been shown that in *age-1* mutants the expression of *sod-3* and *ctl-1/2* increased during oxidative stress, however, in *daf-16* mutants, under the same conditions of hyperoxia, only *sod-3* expression decreased (Yanase et al., 2002). There was a significant upregulation in the expression of *ctl-1* and *ctl-2*. Recently, it was shown that treatment with standardized extract EGb761 from the leaves of *Ginkgo biloba* resulted in decreased expression of stress inducible *ctl* genes in *C. elegans* during oxidative stress and physiological conditions (Kampkötter et al., 2007), and is an important indicator of cellular redox stress. Therefore, a relatively constant expression of catalase upon treatment with basil may reflect an enhanced oxidative stress response and redox balance. Expression of *sod-4* also increased significantly upon treatment with basil. *Sod-4* is an extracellular Cu/Zn SOD that is important in redox balance in the extracellular matrix. However, unlike *sod-3* it has not been identified as a direct downstream target of *daf-16* protein (Murphy et al., 2003).

Since treatment with basil decreased the expression of *daf-2* and *age-1* and upregulated the expression of *daf-16* and *sod-3,* it can be assumed that the phosphorylation cascade, activated by the *daf-2*-ligand interaction was being inhibited. It is also possible that *daf-16* was activated by alternate mechanisms, eg. by sirtuin 2, facilitating its nuclear translocation. *Sir2.1,* which has a human homolog of sirtuin 2 (Sir2) is known to directly activate FOXO under normal energy states (Lamming et al., 2004). This small enzyme can result in life extension similar to what had previously been

seen with caloric restriction (Lamming et al., 2004). However, as discussed previously, the effects of basil on the expression of *sir2.1* were inconclusive, suggesting that the nuclear translocation of *daf-16* was probably due to inhibition in the *daf-2*/AKT/PI_3K mediated phosphorylation. Several protein phosphatases have been identified that can dephosphorylate and inhibit this signaling cascade. In *C. elegans let-92* (protein phosphatase 2A (PP2A)), and its regulatory subunit *pptr-1* are known to dephosphorylate and inactivate AKT thus preventing cytosolic retention and facilitating nuclear translocation *daf-16*/FOXO (Lant & Storey, 2010; Shaw & Dillin, 2009). In response to basil treatments, we observed that expression of *let-92* and *pptr-1* were both upregulated indicating a probable mechanism for inhibiting the AKT/PKB phosphorylation cascade. Moreover, the expression of *ins-7* and *ida-1* positively regulated by the *daf-2* activation were also downregulated upon treatment with basil. The expression of Insulin-7 (*ins-7*) a putative agonist for *daf-2* (Murphy et al., 2007) is positively regulated by the *daf-2* cascade in a feed-forward loop. Its expression is known to be decreased upon activation of *daf-16* mediated transcription (Murphy et al., 2007). Recently *ins-7* RNAi in *C. elegans* was shown to promote life extension by inhibiting *daf-2*, which resulted in increased *daf-16* nuclear entry and increased expression of *sod-3* (Murphy et al., 2007).

Similar to *ins-7*, the expression of *ida-1*(human IA-2 homologue) was also downregulated upon treatment with basil. In *C. elegans ida-1* is expressed in peptidergic neurons, while in mammals (IA-2) expression is extended to pancreatic islet, adrenal medullary and pituitary cells (Zhang et al., 2007). *Ida-1* is thought to play a role in the regulated secretory pathways of neuroendocrine cells such as pancreatic beta cells (Cai et al., 2009). IA-2 has been shown to be induced by insulin (Torii, 2009) and in *C. elegans*

ida-1 causes exocytosis of dense-core vesicles, *ida-1* knockouts have impaired neuroendocrine processes (Torii, 2009) and display lower insulin secretion (Torii, 2009). The *C. elegans daf-28* is similar to mammalian insulin (INS-4) but is expressed in sensory neurons and plays a very important role in neuronal growth and function (Li et al., 2003). However, unlike *ins-7*, expression of *daf-28* is not regulated by *daf-2* and it is also is known to positively regulate the expression of *daf-16* and facilitate its nuclear translocation (Li et al., 2003). An upregulation in expression of *daf-28* in response to basil suggest a possible positive effect on neuronal growth and also an elevation in the *daf-16* mediated stress response in the ASJ and ASN neurons in *C. elegans.*

Insulin Signaling in Response to Oregano

Increased expression and activation of the *C. elegans* insulin/IGF receptor *daf-2* should increase the propagation of the insulin signaling cascade (Prahlad & Morimoto, 2008). This would result in a decreased cellular stress response regulated by *daf-16* but an increase in growth and biosynthetic metabolism (Rafalski & Brunet, 2011). In response to oregano, expression of *daf-2* was moderately upregulated but did not significantly affect the expression of *age-1*. The increased receptors would activate an intrinsic tyrosine kinase upon ligand binding (Lant & Storey, 2010) to promote recruitment of IRS and eventually activation of *age*-1/PI_3K (Barbieri et al., 2003), PDK-1, followed by AKT/PKB (Osaki et al., 2004). Since, active AKT/PKB can decrease nuclear translocation of transcription factor *daf-16*, active insulin signaling cascade should result in decreased expression of genes regulated by *daf-16* (Kwon et al., 2010). As previously discussed, treatment with oregano resulted in increased expression of *daf-16* significantly compared to control. Additionally, the expression of *sod-3* which is

positively regulated by *daf-16* (Murphy et al., 2007) was upregulated several-fold in response to oregano treatment suggesting that *daf-16* was not inhibited by PI3K/AKT. The expression of catalase genes (*ctl*) decreased in response to treatment with oregano. As described previously, reduction in the expression of *ctl* genes may indicate an overall reduction in the ROS accumulation enhancement in redox stress response. Expression of *sod-4* (extracellular $Cu2^{+}/Zn2^{+}$ superoxide dismutase) was decreased in response to oregano. Unlike mitochondrial *sod-3* it has not been identified as being under direct regulation of *daf-16* (Murphy et al., 2003) but is postulated to have signaling functions (Doonan et al., 2008). Extracellular production of H_2O_2 from *sod-4* is thought to traverse the cell membrane and activate insulin signaling by inhibiting the redox sensitive phosphatases (Doonan et al., 2008; Goldstein et al., 2005; Hoogewijs et al., 2008). A downregulation in the expression of *sod-4* may further contribute to the inhibition of the *daf-2* signaling.

It is also possible that *sod-3* expression could be activated by s*ir2.1* (sirtuin) (Lamming et al., 2004) independent of *daf-2* inhibition. However, there was an overall decrease in expression of *sir2.1*seen with oregano treatment, suggesting nuclear translocation of *daf-16* and elevated *sod-3* expression is due to inhibition of PI_3K/AKT mediated phosphorylation. Protein phosphatase *let-92* and its regulatory subunit *pptr-1* are known to inactivate AKT directly and facilitate nuclear translocation *daf-16*/FOXO (Lant & Storey, 2010; Shaw & Dillin, 2009). In response to oregano treatments, we observed a multi-fold increase in expression of *let-92*. Additionally, there was also a significant upregulation of *pptr-1* relative to control, suggesting that a probable mechanism for the inhibition of the AKT/PKB phosphorylation cascade. Moreover, no

change in the expression of *ins-7* and *ida-1*, genes that are positively regulated by *daf-2* (Cai et al., 2009; Murphy et al., 2007) signaling further suggest that there was decreased insulin signaling in *C. elegans* in response to oregano treatment.

However, unlike with basil treatments, the expression of insulin like peptide *daf-28* important for neuronal growth and function (Li et al., 2003) in *C. elegans* was not effected by oregano treatment.

Insulin Signaling in Response to Rosemary

Certain metabolic and environmental conditions result in the activation of *daf-2*/IGF receptor mediated signaling pathway that promotes growth and development, but also decreases cellular stress responses (Lin et al., 2001). When compared to control, treatment with rosemary upregulated expression of *daf-2*. This upregulation in receptors could increase ligand binding allowing for greater insulin receptor substrate recruitment and interaction with *age*-1/PI_3Kinase (Lant & Storey, 2010). *Age-1/*PI_3 K interaction with activated *daf-2* leads to phosphorylation of the membrane lipid PIP_2 enabling the newly formed $PIP_3$to amplify the signaling cascade (Osaki et al., 2004). The amplification by PIP_3 activates PDK1, which phosphorylates and activates the regulatory enzyme AKT/PKB (Barbieri et al., 2003). Active AKT/PKB phosphorylates *daf-16*/FOXO and may cause its cytoplasmic retention. *Daf-16* is a transcription factor regulating the expression of many stress response genes (Kwon et al., 2010). We observed a two-fold increase in *daf-16* expression followed by a significant increase in *sod-3* expression. Since, *sod-3* is a positively regulated by *daf-16*, upregulation of *sod-3* suggests nuclear translocation of active *daf-16* (Hoogewijs et al., 2008). Expression of *ctl* was no different

from control when treated with rosemary, similar to the effect on *ctl* observed with oregano treatment. As discussed previously regulation of *ctl* can involve more than *daf-16*, since it responds to H_2O_2 no change from control possibly reflects a stable redox environment (Henderson et al., 2006). Additionally, expression of extracellular *sod-4* was decreased in response to rosemary. With reduced expression of *sod-4* and formation of H_2O_2 (Doonan et al., 2008; Goldstein et al., 2005; Hoogewijs et al., 2008), the insulin receptor tyrosine phosphatases have been shown to reduce PIP_3 conversion, thereby inhibiting the insulin signaling cascade at PI_3K/AKT (Weinkove et al., 2006).

There is also the possibility that *daf-16* was directly activated by sirtuin 2, which can facilitate its nuclear translocation (Lamming et al., 2004). However, *sir2.1*expression was downregulated in response to rosemary, suggesting that direct activation of *daf-16*/FOXO (Lamming et al., 2004) was not likely the mechanism of action. Based on our results nuclear translocation of *daf-16* was more likely due to the significant downregulation and inactivation of *age-1*/PI_3K. As previously mentioned protein phosphatase such as *let-92*/PP2A and its regulatory subunit *pptr-1*can inactivate AKT (Shaw & Dillin, 2009). In response to rosemary treatments, we observed a downregulation of *let-92* expression, and a marginal difference in *pptr-1* expression from control. This indicates these phosphatases were likely not responsible for preventing the phosphorylation of *daf-16* when treated with rosemary. It is therefore possible that inhibition in PI_3K/AKT is a result of the effect of rosemary extracts further upstream in the signaling cascade. It may be speculated that bioactive compounds in rosemary may be reducing the flux of the *daf-2* signaling by reducing interaction of *daf-2* with its ligand,

reducing PDK-1 activity or inhibiting the intrinsic tyrosine kinase activity, all of which are known targets of natural products (Falasca & Maffucci, 2007; Spencer et al., 2003).

Furthermore, genes that are positively regulated by *daf-2* activation such as *ins-7* and *ida-1* (Cai et al., 2009; Murphy et al., 2007) showed little change from control upon rosemary treatment, further substantiation to decreased insulin signaling. However, expression of *daf-28* (*C. elegans* homologue for INS-4) is not regulated by *daf-2* (Li et al., 2003), but shown to positively regulate *daf-16* expression and transcription (Li et al., 2003) was upregulated in response to rosemary treatment. In *C. elegans* the response to rosemary treatment could possibly increase neuronal growth and development with added neuroprotective *daf-16* controlled antioxidant production.

Insulin Signaling in Response to Sage

Daf-2/IGF receptor when bound to its ligand activates a cascade resulting in growth, fertility, and metabolic activity, alternatively unbound *daf-2* leads to greater longevity thru an upregulated cellular stress response (Rafalski & Brunet, 2011). When compared to control, treatment with sage increased expression of *daf-2.* It is possible that this upregulation in receptors could promote insulin receptor substrate interaction with *age*-1/PI_3Kinase (Lant & Storey, 2010). However, unlike *daf-2*, we did not see a significant change in *age-1/*PI_3 K expression upon treatment with sage, leaving the interaction with elevated *daf-2* hard to determine. *Age-1*/PI_3K phosphorylates the lipid membrane PIP_2 forming the second messenger PIP_3 (Osaki et al., 2004) which activates PDK1, a kinase that can phosphorylate and activate the regulatory enzyme AKT/PKB (Barbieri et al., 2003). It is well known that active AKT promotes cytosolic retention of

transcription factor *daf-16*/FOXO (Henderson et al., 2006). Even though we saw an increase in expression of *daf-2*, we also observed a significant increase in *daf-16* expression over control when treated with sage. However, there was not a significant change in *sod-3* expression. In *C. elegans* and other eukaryotes, FOXO transcriptionally activates the expression of *sod-3* (Hoogewijs et al., 2008). Basal expression of *sod-3* in response to sage treatment may either suggest a reduced nuclear translocation of *daf-16*, and/or post-transcriptional/translational degradation of *sod-3* due to reduced oxidative mitochondrial oxidative stress. Additionally, similar to the effects on *ctl* observed with oregano, and rosemary, expression of *ctl* was also not different from control when treated with sage, further suggesting low transcriptional activation of *ctl* expression by *daf-16* and/or ROS (Henderson et al., 2006). The expression of extracellular SOD (*sod-4)* was also decreased in response to sage, suggesting a lower formation of H_2O_2 and therefore a reduced activation of the insulin signaling (Doonan et al., 2008; Goldstein et al., 2005; Hoogewijs et al., 2008; Weinkove et al., 2006).

Our results indicated a multi-fold increase in *sir2.1* expression in response to sage, as *daf-16* activity is also regulated by sirtuin 2/*sir2.1* (Lamming et al., 2004). It is likely that increased *sir2.1* expression may activate *Daf-16*/FOXO independent of AKT, resulting in further increase in expression of genes downstream of *daf-16*. However, as discussed above, relatively constant levels of *sod-3* and *ctl* suggest this was not the case. Nevertheless, phenolic compounds (eg. resveratrol) have previously been shown to be effective at increasing sirtuin expression (Lamming et al., 2004). As, increased sirtuin activity has been associated with reduced amyloid β accumulation in Alzheimer's disease (Sun et al., 2010), independently reducing metabolism, promoting stress response and

enhancing lifespan. We therefore anticipate to further investigate biological functionality of sage in this regard (Akhondzadeh et al., 2003; Iuvone et al., 2006; Mohsen et al., 2006).

In response to sage treatments, we observed an upregulation of *let-92* expression, and unchanged *pptr-1* expression from control suggesting a possible phosphatase mediated inhibition in the *daf-2* signaling (Shaw & Dillin, 2009). Differential results were observed in *ins-7* and *ida-1,* two genes that are positively regulated by activated *daf-2* (Cai et al., 2009; Murphy et al., 2007). *Ins-7* was significantly upregulated from control upon sage treatment and *ida-1* was downregulated. Moreover, expression of a positive regulator of *daf-16* function, *daf-28/*INS-4 (Li et al., 2003) increased suggesting a homeostatic modulating activity of sage in *C. elegans* on *daf-16* and *daf-2* mediated pathways, with possible beneficial effects on stress response and growth/metabolism.

Insulin Signaling in Response to Thyme

Increased stress response and longevity is typically associated with reduced activation of *daf-2*/IGF, mediated via *age-1*/PI_3K and AKT/PKB signaling (Weinkove et al., 2006). When compared to control, treatment with thyme significantly downregulated expression of *daf-2. Age-1/*PI_3 K expression was also downregulated in response to thyme. However, we observed an increase in *daf-16, sod-3* and *ctl* expression, suggesting an absence of AKT/PKB mediated inhibition of *daf-16* nuclear translocation thus enhancing the redox stress response (Henderson et al., 2006; Hoogewijs et al., 2008). Moreover, a decrease in the expression of extracellular *sod-4* further suggests a decrease

in insulin signaling by inactivation of phosphatases (Doonan et al., 2008; Goldstein et al., 2005; Hoogewijs et al., 2008; Weinkove et al., 2006).

Sirtuin 2 has also been shown to directly activate *daf-16* inducing nuclear translocation and transcription of its target genes (Lamming et al., 2004). However, the results for *sir2.1/*sirtuin 2 expression when treated with thyme relative to control were inconclusive. In response to thyme treatments, we also observed little change in expression of *let-92* or *pptr-1* suggesting a possible non phosphatase mediated inhibition in *daf-2* signaling either by effecting interaction of *daf-2* with its ligands, or *age-1*/PI_3K activity (Falasca & Maffucci, 2007; Spencer et al., 2003).

The expression of *ins-7* and *ida-1,* genes that are positively regulated by *daf-2* were also not effected by thyme treatment (Murphy et al., 2007). This indicated the insulin signaling cascade was not activated (Murphy et al., 2007) above basal levels. Similarly, expression of *daf-28/*INS-4 (expressed in *C. elegans* sensory neurons) shown to positively regulate *daf-16* activity (W. Li et al., 2003) was downregulated relative to control, indicating that thyme treatments did not have any direct *daf-2* independent neuronal or neuroendocrine benefit.

Ranking of Lamiaceae Herbs by Effectiveness

Using linear regression from the RF data generated the five herbs were ranked according to the effect on expression of genes critical for ILS at each concentration (Table 8.). We calculated the vector of the slope and the intercept from the equation of the curve (Table 7). The slope will provide information on effective concentrations (Table 9.), while the intercept will provide most effective herb (Table 9.). The vector of

the slope tells us about the trend of the treatment. If the slope is negative lower concentrations are more effective, if the slope is positive the higher concentrations are more effective. The magnitude of the intercept can be used to determine the overall effect of an herb. The treatment with the highest intercept (> 1) provides the most effective upregulation in expression, while the treatment with the lowest intercept (< 1) affords most effective downregulation in gene expression.

Table 7. Dose dependent response of *Lamiaceae* herb treatment on expression of genes relevant to insulin signaling by slope and Y-intercept (Y-Intc).

	Basil		**Oregano**		**Rosemary**		**Sage**		**Thyme**	
GENE	**Slope**	**Y-Intc**	**Slope**	**Y-Intc**	**Slope**	**Y-Intc**	**Slope**	**Y-Intc**	**Slope**	**Y-Intc**
daf-16a	-0.031	1.876	-0.003	1.346	-0.004	1.346	0.020	1.159	-0.039	1.611
daf-16	-0.009	0.250	0.007	1.670	0.003	2.053	-0.019	1.639	0.003	2.610
daf-2	-0.019	0.876	0.004	1.153	-0.025	1.311	0.015	1.401	-0.007	0.729
age-1	-0.022	0.940	-0.001	0.945	0.009	0.707	-0.003	0.925	-0.021	0.986
pp2A	0.002	1.083	-0.069	4.582	-0.022	0.910	-0.003	3.679	0.037	0.595
pptr-1	0.014	0.947	0.008	1.393	-0.001	0.781	-0.008	1.175	-0.004	0.862
sod-3	-0.067	1.693	-0.083	2.035	-0.001	1.725	0.006	0.757	-0.018	1.441
sod-4	0.025	1.030	0.015	0.671	-0.015	0.921	-0.046	1.012	-0.009	0.872
ctl	-0.010	0.911	0.020	0.793	0.011	0.898	-0.010	1.127	0.000	1.431
sir2.1	-0.004	0.537	-0.001	0.469	-0.005	0.303	-0.002	4.110	0.041	0.658
sir2.1	0.009	1.131	0.019	0.901	-0.002	0.813	-0.029	1.185	-0.005	0.850
ins-7	-0.000	0.971	0.017	0.953	-0.004	1.064	-0.031	0.849	-0.003	0.926
ins-4	0.031	1.297	-0.024	0.952	0.001	1.548	0.043	0.948	0.020	0.517
ida-1	-0.001	0.848	0.014	0.867	-0.020	1.012	-0.000	1.198	-0.007	0.923

Table 8. Ranking of *Lamiaceae* herbs based on Y-intercept values. Unshaded cells represent upregulation, shaded boxes represent downregulation.

Ranking of Different *Lamiaceae* Herbs					
Upregulation	**1**	**2**	**3**	**4**	**5**
Downregulation	**5**	**4**	**3**	**2**	**1**
daf-16a	Basil	Oregano	Rosemary	Sage	Thyme
daf-16	Thyme	Rosemary	Oregano	Sage	Basil
daf-2	Sage	Rosemary	Oregano	Basil	Thyme
age-1	Thyme	Oregano	Basil	Sage	Rosemary
let-92	Oregano	Sage	Basil	Rosemary	Thyme
pptr-1	Oregano	Sage	Basil	Thyme	Rosemary
sod-3	Oregano	Rosemary	Basil	Thyme	Sage
sod-4	Basil	Sage	Rosemary	Thyme	Oregano
ctl	Thyme	Sage	Basil	Rosemary	Oregano
sir2.1	Sage	Thyme	Basil	Oregano	Rosemary
sir2.1	Sage	Basil	Oregano	Thyme	Rosemary
ins-7	Rosemary	Basil	Oregano	Sage	Thyme
daf-28	Rosemary	Basil	Sage	Oregano	Thyme
ida-1	Rosemary	Sage	Thyme	Oregano	Basil

Table 9. Based on vector of the slope/intercept data the overall most effective herb resulting in upregulation/downregulation of each gene at the lowest concentration could be identified.

Lowest Concentration for Insulin Signaling Pathway Modulation				
Gene	**Most Effective**	**Upregulation**	**Most Effective**	**Downregulation**
daf-16a	Basil	1 mg/ml	-	-
daf-16	Thyme	1 mg/ml	Basil	10 mg/ml
daf-2	Sage	10 mg/ml	Thyme	10 mg/ml
age-1	-	-	Rosemary	1 mg/ml
let-92	Oregano	1 mg/ml	Thyme	1 mg/ml
pptr-1	Oregano	5 mg/ml	Rosemary	1mg/ml
sod-3	Oregano	1 mg/ml	Sage	1 mg/ml
sod-4	Basil	10 mg/ml	Oregano	1 mg/ml
ctl	Thyme	5 mg/ml	Oregano	1 mg/ml
sir2.1	Sage	1 mg/ml	Rosemary	10 mg/ml
sir2.1	Sage	5 mg/ml	Rosemary	10 mg/ml
ins-7	Rosemary	1 mg/ml	Thyme	10 mg/ml
daf-28	Rosemary	1 mg/ml	Thyme	1 mg/ml
ida-1	Rosemary	1 mg/ml	Basil	10 mg/ml

Environmental and dietary stress can induce changes in the cellular redox status, resulting in genetic mutations or abnormalities in the ILS pathway. Lamiaceae herbs are well known for therapeutic potential, we have established dose-dependent responses to these herbs in the ILS pathway, and how the responses could effect signaling in dysregulation. The preliminary results have indicated that rosemary treatment ranked the highest on total number of genes modulated at the lowest downregulation or highest upregulation with 7 (Table 8-9). Following rosemary was thyme with 6, oregano with 5, then sage and basil with 4 each (Table 8-9). Another important aspect of this data is we offer a comparison of similarities and differences between herbs according to gene response (Table 7-8) this could provide an insight on how frequent there is synergy between herbs. Other useful evidence that can be extrapolated from the data is multiple examples of upregulation and downregulation within the same treatment and gene, simply at different concentrations.

Conclusion

Based on our study results and survey of literature we hypothesize that dietary *Lamiaceae* herbs can modulate other important pathways in eukaryotic systems that regulate stress response signaling. Dietary *Lamiaceae* herbs that can positively modulate different stress response signaling may prove to be beneficial in management of diseases that can result from their dysregulation.

Although we continued to evaluate the effect of all of these *Lamiaceae* herbs on the Nrf2/ARE, MAPK, HIF, Hsp, UPR, TGF-β, and apoptosis signaling pathways, only the results for the best performing herb for modulation of key aspects of

neurodegenerative disease were reported here after. Thus, due to the dose dependent *in vivo* effects we observed on insulin signaling in response to sage we determined that this *Lamiaceae* herb would be most effective at attenuation of neurodegeneration.

CHAPTER II

SALVIA OFFICINALIS MODULATES NRF2/ARE (*SKN-1*/ARE) GENE EXPRESSION IN *C. ELEGANS*

Introduction

Nrf2/ARE Signaling Pathway

Oxidative stress driven neuroinflammation has been implicated in the development of Alzheimer's disease (AD), Parkinson's disease (PD), and other neurodegenerative processes (Glass et al., 2010; Sykiotis et al., 2011). Consequently, targeting oxidative stress and neuroinflammation has been proposed as a suitable early treatment to manage neurodegenerative pathologies (Calabrese et al., 2008; Lin & Beal, 2006; Malkus et al., 2009). At a cellular level protection against oxidants and inflammatory signals is achieved via the expression of antioxidant and anti-inflammatory proteins (Jung & Kwak, 2010; Kelsey et al., 2010; Matteo et al., 2003). These genes are regulated by a coordinated activity of several important transcription factors including Nrf2 (nuclear factor E2-related factor 2) (Jung & Kwak, 2010; van Muiswinkel & Kuiperij, 2005). Nrf2 is a nuclear Cap'n'Collar basic region-leucine zipper (bZip) transcription factor that controls the expression of many antioxidant and detoxification genes (Phase II) via binding to the promotor region called the antioxidant response element (ARE) present upstream of the coding region (Kaspar et al., 2009; Leiser & Miller, 2010; Luo et al., 2011; Ramsey et al., 2007).

In homeostatic cellular environments Nrf2 is retained cytoplasmically by kelch like-ECH-associated protein-1 (Keap-1) (Kaspar et al, 2009) (Figure 2). Oxidative and electrophilic stress, or phosphorylation by kinases such as protein kinase C (PKC) (Kang et al., 2005; Kaspar et al., 2009), phosphatidyl inositol-3 kinase (PI_3K), and mitogen activated protein kinase (MAPK) disrupt keap-1(ubiquitin marker on Nrf2) and promotes the dissociation of Nrf2 and keap-1(Kang et al., 2005) (Figure 2). Unbound Nrf2 accumulates and translocates into the nucleus where it binds to the ARE to effect gene transcription (Jaiswal, 2010; Nguyen et al., 2003). Currently, over 200 genes important in antioxidant and anti-inflammatory response have been identified to be regulated by the Nrf2/ARE system. A known direct downstream transcript is the iron sulfur proteins (ISP), which facilitate redox reactions in mitochondrial electron transport (Paupe et al., 2009) (Figure 2). Metallothiones (MTL) which bind to metals and manage cellular oxidative stress are also regulated by Nrf2/ARE (Thirumoorthy et al., 2011) (Figure 2). Expression of several proteins involved in glutathione (GSH) metabolism is also driven by the Nrf2/ARE system. These include the peptide glutathione (GSH), glutathione reductase (GSR), glutathione synthestase (GSS), and glutathione-s-transferase (GST) important in glutathione mediated neutralization of electrophilic compounds (Harvey et al., 2009; Jung & Kwak, 2010) (Figure 2). Additionally, the Nrf2/ARE system also regulates the function of proteins whose expression is not directly dependent on Nrf2. These include glutathione dependent antioxidant proteins like glutaredoxin (GLRX), which are maintained in their active reduced state by GSH (Holmgren & Fernandes, 2004) (Figure 2).

Neurodegeneration and the Nrf2/ARE Pathway

Oxidative stress and neuroinflammation has been identified in dopaminergic neurons in several animal models of neurodegneration (Holtz, Turetzky, Jong, & O'Malley, 2006; Kahle, Waak, & Gasser, 2009; She et al., 2011). Inhibition of oxidative stress as a result of elevated COX II activity has been shown to prevent degradation of dopaminergic neurons upon exposure to the neurotoxic compound MPP^+ (Mazzio et al., 2011). This inhibition was primarily driven by elevated expression and inhibition of genes driven and regulated by Nrf2/ARE system. In *C. elegans* model activation of *skn*-1/Nrf2 in the ASI neurons by caloric restriction has shown to increase lifespan (Sykiotis et al., 2011). Similarly, a reduction in inflammatory markers via resveratrol and caloric restriction has been shown to confer protection against neuronal degradation in rodents (Sun et al., 2010). Consequently, there is great interest in developing dietary or pharmacological strategies to manage neurodegenerative diseases via this approach.

Sage as Modulator of Antioxidant Stress Response

Salvia Officinalis (sage) is a common *Lamiaceae* herb well known for its health promoting effects in traditional herbal medicine. It has been recommended as an agent to improve mood, reduce stress, and enhance cognitive abilities in humans (Kennedy et al., 2006, 2011; Scholey et al., 2008). Recent research has also indicated that dietary ingestion of sage increases protection against oxidants via activation of several antioxidant defense systems (Lima et al., 2006; Sá et al., 2009; Yoo et al., 2008). Even though the mechanism of these protective abilities is not clearly understood several bioactive compounds such as carnosic acid, carnosol, rosmanol and caffeic acid are

thought to be responsible (Dorman et al., 2003; Matsingou et al., 2003). Here we have evaluated the potential effect of sage on the expression and activity of Nrf2/ARE dependent genes in *C. elegans* model as a possible approach for prevention and management of neurodegenerative diseases.

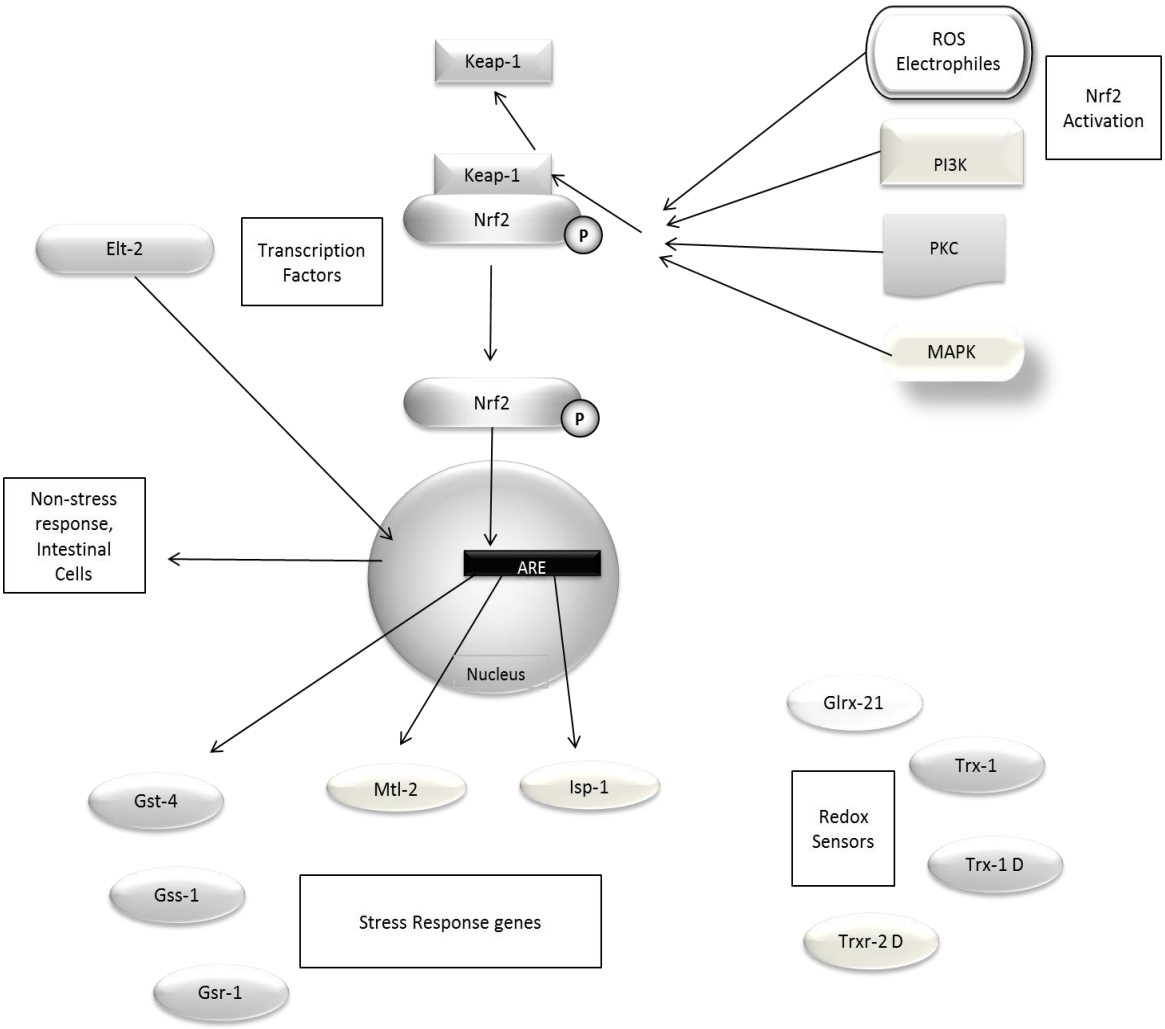

Figure 2. Nuclear factor-erythroid-2 related factor-2 (Nrf2) signaling via transcription of genes in the prompter region of the antioxidant response element (ARE). While expression of genes that function as redox sensors were also evaluated.

Methods

C. elegans with GFP promoter constructs of genes relevant to *skn-1*/ARE (Human Nrf2/ARE) pathway (Table 10) obtained from the Caenorhabditis Genetics Center were used to study the effect of sage on the expression and activity of genes regulated by *skn-1*/Nrf2/ARE system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 10. List of *C. elegans* genes evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org**.**

SKN-1			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
LG326	***skn-1***	Nuclear factor-erythroid-2 related factor-2 (Nrf2) transcription factor	WBGene00004804
MR142	***elt-2***	GATA4-6 transcription factors-primarily intestinal	WBGene00001250
MR164	***elt-2***	GATA4-6 transcription factors-primarily intestinal	WBGene00001250
OH7631	***elt-2***	GATA4-6 transcription factors-neuronal	WBGene00001250
BC14279	***isp-1***	Iron sulfur protein (ISP)	WBGene00002162
CL2122	***mtl-2***	Metallothioneins (MTL)	WBGene00003474
CL2120	***mtl-2***	Metallothioneins (MTL)	WBGene00006789
BC11072	***gsr-1***	Glutathione diSulfide Reductase (GSR)	WBGene00008117
BC12667	***gss-1***	Glutathione synthetase (GSS)	WBGene00010941
BC13348	***gst-4***	Glutathione S-Transferase (GST)	WBGene00001752
CL2166	***gst-4***	Glutathione S-Transferase (GST)	WBGene00001752
BC12039	***glrx-21 D***	Glutaredoxin (GLRX) driven (D)	WBGene00022663
OE3010	***trx-1***	Thioredoxin (TRX)	WBGene00015062
BC13081	***trx-1 D***	Thioredoxin (TRX) driven (D)	WBGene00015062
BC12017	***trxr-2 D***	Thioredoxin reductase (TRXR) driven (D)	WBGene00014028

Results

Effect of Sage on Nrf2/ARE Signaling

An overall downregulation in *skn-1* expression was observed in response to sage treatment. The lowest decrease in expression was seen at 5 mg/ml (RF= 0.702, *p= 0.010*), followed by 1 mg/ml (RF= 0.864, *p= 0.002*), and no significant difference from control at 10 mg/ml. The first of two strains evaluating *elt-2* (MR142) primarily expressed in the intestine was no different from control at 1 mg/ml, 5 mg/ml, and 10 mg/ml sage treatment. The second *elt-2* (MR164) expressing strain was significantly downregulated over control in a dose dependent manner, with the lowest values at 10 mg/ml (RF= 0.662, *p <0.05*), 5 mg/ml (RF= 0.780, *p= < 0.05*) and 1 mg/ml (RF= 0.919, *p= < 0.05*). While the *elt-2* (OH7631) strain expressed for neuronal differentiation was upregulated multifold over control at 5 mg/ml (RF= 2.522, *p= 0.001*), and 10 mg/ml (RF= 3.024, *p= < 0.05*), followed by significant increase at 1 mg/ml (RF= 1.415, *p= < 0.05*). The response to sage treatment on *isp-1* gene expression resulted in an overall upregulation compared to control. The highest increase was seen at 1 mg/ml (RF= 1.340, *p= 0.002*), followed by moderate increases at 5 mg/ml (RF= 1.174, *p= < 0.05*), and 10 mg/ml (RF= 1.158, *p= <0.05*). When compared to control expression of the metallothionein coding *mtl-2* (CL2122) was not significantly different at 1 mg/ml or 5 mg/ml, but a significant downregulation was observed at 10 mg/ml (RF= 0.636, *p= 0.028*). While the other strain expressing *mtl-2* (CL21220) was only significantly downregulated at 1 mg/ml (RF= 0.937, *p= < 0.05*) sage, expression at 5 mg/ml and 10 mg/ml were not statistically different from control. Overall *gsr-1* expression was upregulated in response to sage, with the highest upregulation seen at 5 mg/ml (RF= 1.360, *p= 0.001*), then 10 mg/ml (RF=

1.223, *p= 0.028*), and 1 mg/ml (RF= 1.170, *p= 0.055*). Gene expression of glutathione synthetase coding gene *gss-1* was only slightly lower than control at 1 mg/ml (RF= 0.997, *p= 0.006*), and 5 mg/ml (RF= 0.988, *p= 0.015*), but no different from control at 10 mg/ml of sage treatment. The expression of *gst-4* in two strains was upregulated overall. The first strain (BC13348) was upregulated in expression at only 1 mg/ml (RF= 1.365, *p= < 0.05*), and 5 mg/ml (RF= 1.166, *p= 0.003*), but no difference from control was seen at 10 mg/ml of sage treatment. The second strain was approaching multifold upregulations at 1 mg/ml (RF= 1.960, *p= 0.001*), and 5 mg/ml (RF= 1.946, p= < 0.05) followed by 10 mg/ml (RF= 1,749, *p= < 0.05*).

The expression of *glrx-21* driven genes was not significantly different from control at 1 mg/ml and 5 mg/ml while only slightly increased at 10 mg/ml (RF= 1.048, *p= 0.001*). Relative to control in response to sage, the expression of the thioredoxin coding *trx-1*at 1 mg/ml (RF= 1.064, *p= 0.002*), and 5 mg/ml (RF= 1.058, *p= 0.006*) was slightly above control, while a higher increase was seen at 10 mg/ml (RF= 1.212, *p= 0.001*). There was a multifold upregulation in *trx-1* driven gene expression at 1 mg/ml (2.562, *p= 0.006*), and 10 mg/ml (RF= 2.169, *p= < 0.05*), followed by a near two fold increase at 5 mg/ml (RF= 1.948, *p= 0.015*). A significant downregulation was only seen at 1 mg/ml (RF= 0.875, *p= < 0.05*) for *trxr-2*, no change compared to control was seen at 5 mg/ml, and 10 mg/ml.

Table 11. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Sage Concentration								
	1mg/ml			5mg/ml			10mg/ml		
	RF	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
skn-1	0.864	0.031	***0.002***	0.702	0.103	***0.010***	0.754	0.118	*0.125*
elt-2	1.169	0.227	*0.192*	1.179	0.400	*0.318*	1.118	0.437	*0.389*
elt-2	0.919	0.098	***< 0.05***	0.780	0.049	***< 0.05***	0.662	0.034	***< 0.05***
elt-2	1.415	0.065	***< 0.05***	2.522	0.332	***0.001***	3.024	0.120	***< 0.05***
isp-1	1.340	0.215	***0.002***	1.174	0.087	***< 0.05***	1.158	0.083	***< 0.05***
mtl-2	1.025	0.287	*0.085*	0.856	0.161	*0.375*	0.636	0.172	***0.028***
mtl-2	0.937	0.143	***< 0.05***	0.676	0.054	*0.353*	0.650	0.085	*0.465*
gsr-1	1.170	0.091	***0.055***	1.360	0.096	***0.001***	1.223	0.103	***0.028***
gss-1	0.997	0.068	***0.006***	0.988	0.061	***0.015***	0.945	0.125	*0.384*
gst-4	1.365	0.046	***< 0.05***	1.166	0.044	***0.003***	1.089	0.043	*0.165*
gst-4	1.960	0.305	***0.001***	1.946	0.124	***0.000***	1.749	0.167	***< 0.05***
glrx-21 D	0.889	0.070	*0.266*	0.986	0.138	*0.273*	1.048	0.050	***0.001***
trx-1	1.064	0.066	***0.022***	1.058	0.049	***0.006***	1.212	0.060	***0.001***
trx-1 D	2.562	0.469	***0.006***	1.948	0.349	***0.015***	2.169	0.189	***< 0.05***
trxr-2 D	0.875	0.066	***< 0.05***	1.108	0.074	*0.251*	1.068	0.041	*0.170*

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

Nrf2/ARE Signaling in Response to Sage

The expression of *skn-1*/Nrf2 decreased in response to sage treatment. In previous studies it has been noted that changes in protein expression after treatment with black tea polyphenols did not necessarily indicate a change in mRNA levels as Nrf2/ARE response can also be activated by posttranslational modification such as phosphorylation (Patel & Maru, 2008).

The expression of transcription factor GATA (*elt-2* in *C. elegans),* a gene downstream of *skn-1*/Nrf2/ARE was inconclusive. In two of the three strains evaluated *elt-2* was expressed primarily in intestines (Mcghee et al., 2010). Where the expression of *elt-2* was either no different or was lower than control upon treatment with sage. Interestingly, in the third strain where expression of *elt-2* is also driven by the gene *nhr-67* there was an increased response to sage treatment. *Nhr-67* is neuronally expressed and involved in gustatory neuron development (Sarin et al., 2009). These results possibly indicate that there was a tissue specific response to sage extract and perhaps enhanced neuronal development. This could be due to an elevated stress response, and thus a lowered level of oxidative or electrophilic stress. As it has been shown neuronal proliferation and survival is more likely to occur in a reducing environment (Calabrese et al., 2008; Rafalski & Brunet, 2011; Shih et al., 2003).

In response to sage treatments the expression of *isp-1* was significantly upregulated. *Isp-1* is a mitochondrial protein involved in respiration and electron transport. Our results indicate that in response to sage treatment there was either an increase in total mitochondrial numbers or an increase in mitochondrial respiration. However, the expression of another gene *mtl-2*, which is also under the transcriptional regulation of Nrf2/ARE system decreased in response to sage treatment. Studies have previously indicated that increased redox stress in response to heavy metals glucocorticoids, inflammatory signals can increase the expression of *mtl-2* gene to reduce oxidative stress (Bi et al., 2004; Mocchegiani et al., 2000). Therefore, a downregulation in *mtl-2* gene in conjugation to increased ISP expression suggest that even though there was an elevated mitochondrial respiration it did not result in increased redox stress in the

cellular environment. This could probably be due to an enhanced antioxidant defense response upon treatment with sage.

When we evaluated the effect of sage treatment on GSH metabolism we observed that there was no change in the expression of *gss-1* gene. As noted above GSS is the rate limiting enzyme in the biosynthesis of glutathione peptide. However, the expression of *gsr-1* which codes for glutathione reductase was upregulated in response to sage treatment. Our results suggest that even though there was no increase in GSH biosynthesis in response to sage treatment, there was a more efficient regeneration of GSSH to its reduced form of GSH by *gsr-1*. In addition, treatment with sage also resulted in upregulation of *gst-4*. As described previously GST is transcriptionally regulated by Nrf2 and is involved in the detoxification of electrophilic species. Together these results suggest an enhanced GSH mediated redox response upon treatment with sage.

This can be further corroborated based on the effect of sage on the expression of the gene *glrx-21*. GLRX utilizes the reducing power of GSH to catalyze the reversible reduction of protein disulfides and is a very sensitive indicator of redox environment in the cell (Morgan et al., 2010). In response to sage treatment expression of *glrx-21* did not change suggesting that there was no increase in cellular oxidative stress. In addition we also noted a multi-fold increase in the expression of *trx-1* gene. TRX like GLRX is also involved in maintenance of cellular redox environment, and is known to increase the binding of *skn-1*/Nrf2 to the ARE regions of several genes (Hansen et al., 2004). Therefore an increased expression in response to sage treatment may indicate a more efficient management of cellular redox environment and also an increased binding Nrf2 binding of Nrf2 to ARE resulting in changes in gene transcription. Furthermore, the

expression of TRX driven genes also increased two-fold in response to sage treatment which further indicates an increased *trx-1* transcriptional activity in response to treatment with sage. The expression of *trxr-1* was downregulated in response to sage treatment. Unlike GLRX which is regenerated by using the reducing power of GSH, oxidized TRX is reduced by TRXR (M.-ra Kim et al., 2003). A downregulation in TRXR gene indicates a stable redox environment, with much of its TRX in its active reduced form.

Conclusion

Treatment with sage did not directly increase the expression of transcription factor *skn-1*/Nrf2. However the expression of several genes involved in GSH metabolism were upregulated in response to sage treatment which indirectly indicated that there was an increased transcriptional activity of *skn-1*/Nrf2 by its translocation into the nucleus and interaction with ARE and several related genes. We also noted that there was an increase in mitochondrial respiration and mitochondrial numbers. However this did not result in an increase in the oxidative environment of the cell. As indicated by either upregulation or no change in the levels of important redox sensing proteins in the cell.

CHAPTER III

THE *IN VIVO* EFFECT OF *SALVIA OFFICINALIS* ON THE MAPK SIGNALING CASCADE

Introduction

The MAPK Signaling Cascade

In neuronal and non-neuronal cells, (mitogen activated kinase) MAPK signaling cascades control several cell function such as differentiation, death and survival (Takeda & Ichijo, 2002). These pathways are evolutionarily well conserved in eukaryotic cells and are classified into three different cascades. Namely, the extra cellular signal-regulated kinases (ERKs), c-jun-N-terminal kinases (JNKs), and the p38 MAPKs (Takeda & Ichijo, 2002). The ERK cascade is generally involved in the control of cell proliferation and differentiation by mitogens and growth factors, while the JNK and p38 regulated cascades are favorably activated by environmental stress such as UV radiation, X-rays, heat shock, osmotic shock, and pro-inflammatory cytokines (Takeda & Ichijo, 2002). Although strongly regulated by oxidative stress, this pathway also plays an important role in immunity (Cuadrado & Nebreda, 2010).

Regulation and Dysregulation of the MAPK Cascade

Recently, p38 MAPKs have been implicated in induction in neuronal apoptotic pathways important in neuronal development and neuronal pathologies (Takeda & Ichijo, 2002). Over expression of p38 in microglial cells has been shown to contribute to

amyloid β induced inflammation in Alzheimer's disease (AD). In astrocytes overactive p38 MAPK has been shown to increase inflammation and excitotoxicity. On the other hand neuronal p38 MAPK signaling has been attributed to hyperphosphorylated microtubule associated tau proteins (Li et al., 2003; Munoz & Ammit, 2010). Emerging evidence has shown that declines in p38 signaling are seen with aging and has been linked to reduced immunocompetency and increased inflammation in rodent models (Youngman, Rogers, & Kim, 2011).

MAPK signaling has also been implicated in axonal regeneration and degeneration, reduction in signaling reduces distal degeneration and inhibits proximal neurite growth (Nix et al., 2011). Furthermore, a coordinated MAPK action and MAPK phosphatase-mediated inactivation response between JNK and p38 was indicated to stimulate axon regeneration (Nix et al., 2011). The coordination, duration and intensity of the MAPK signaling is critically regulated by phosphastases that deactivate or reduce the flux of the MAPK cascade after beneficial effects have been maximized (Cuadrado & Nebreda, 2010). This regulation by phosphatases includes the action of protein phosphatase 2A (PP2A). Reduced or dysregulated activity of protein phosphatases has also been linked to neurodegenerative pathologies via improper neurotransmitter transport and hyperphosphorylation of tau proteins. (Gong & Iqbal, 2008; Zhu et al., 2005). In *C. elegans* this pathway was shown to increase resistance against neurotoxic methamphetamines and induction of dopamine induced Parkinson's disease (PD) symptoms (Schreiber & McIntire, 2011).

Consequently, regulation of MAPK signaling has been become an important target for development of therapeutic compounds for neurodegenerative pathologies. As

indicated previously, natural products derived bioactives have recently been shown to have beneficial effects against a range of neurodenerative diseases (Kannappan et al., 2011). Here we have evaluated the effect of sage treatment on expression of genes related to the MAPK signaling cascade with possible links to neuroprotection. A number of *in vitro* (Iuvone et al., 2006), *in vivo* animal (EL-Kholy et al., 2010) showed positive results, and double-blind placebo-controlled studies in healthy humans (Scholey et al., 2008) as well as studies in AD patients treated with sage resulted in improved memory, attention, motor function, and mood.

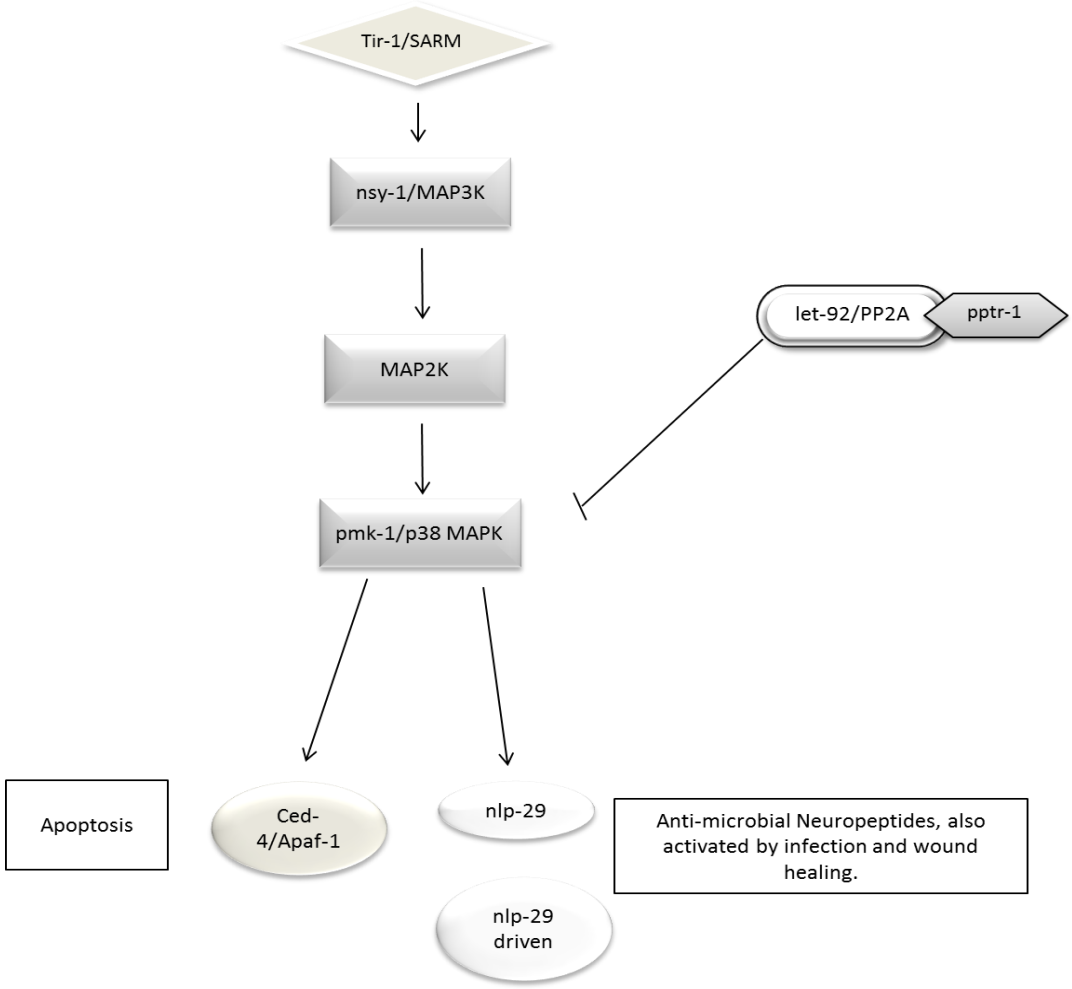

Figure 3. Mitogen activated kinase signaling via phosphorylation cascade with *C. elegans* and human identifiers.

Methods

C. elegans with GFP promoter constructs of genes relevant to MAPK signaling pathway (Table 12) obtained from the *Caenorhabditis* Genetics Center were used to study the effect of sage on the expression and activity of genes regulated by the MAPK system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 12. List of *C. elegans* genes evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Mitogen Activated Protein Kinase Signaling Cascade			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
BC11466	***tir-1***	Toll-interleukin 1 receptor domain-containing protein SARM	WBGene00006575
BC10545	***nsy-1***	(MAP3K) Apoptosis signal-regulating kinase (ASK)	WBGene00003822
DA1750	***pmk-1***	(MAPK) Human p38 mitogen-activated protein kinase	WBGene00004055
IG692	***nlp-29***	Antimicrobial, neuropeptide-like protein	WBGene00003767
IG274	***nlp-29 D***	Antimicrobial, neuropeptide-like protein driven (D)	WBGene00003767
BC10950	***let-92***	Protein Phosphatase 2A	WBGene00002363
BC14613	***pptr-1***	Protein Phosphatase 2A regulatory subunit	WBGene00012348
WS5770	***ced-4***	Apoptotic protease activating factor 1 (Apaf-1)	WBGene00000418

Results

Effect of Sage on the MAPK Signaling Cascade

Significant upregulations were observed when evaluating *tir-1* (BC11466) gene expression in response to sage at 1 mg/ml (RF= 1.097, *p= 0.003*), 5 mg/ml (RF= 1.203, *p= <0.05*), and 10 mg/ml (RF= 1.208, *p= < 0.013*). Near multifold increases in *nsy-1* (BC10545) gene expression were seen at 1 mg/ml (RF= 1.364, *p= <0.05*), 5 mg/ml (RF= 1.758, *p= <0.05*), and 10 mg/ml (RF= 1.572, *p= 0.002*). The gene expression of *pmk-1* (DA1750) in response to sage was upregulated at 5 mg/ml (RF= 1.718, *p= 0.006*), and 10 mg/ml (RF= 1.843, *p= 0.032*), but was no different from control at 1 mg/ml. There was increased expression of *let-92* in response to sage across all test concentrations [1 mg/ml (RF=3.84, p=<0.05), 5 mg/ml (RF= 3.37, *p=0.004*), and 10 mg/ml (RF=3.78, *p=<0.05*)] compared to control expression. The catalytic subunit *pptr-1* was increased significantly at 1 mg/ml (RF=1.26, *p=<0.05*), and 10 mg/ml (RF=1.17, *p=<0.05*), but 5 mg/ml of sage was slightly downregulated relative to control. Downregulations were seen in response to sage treatments for *nlp-29* (IG692) gene expression at 1 mg/ml (RF=0.928, *p= 0.007*), and 10 mg/ml (RF= 0.619, *p= 0.017*), with no change from control at 5 mg/ml. Expression of *nlp-29 driven* (IG274) genes were significantly downregulated at 1 mg/ml (RF=0.571, *p=0.003*), and at 10 mg/ml (RF= 0.385, *p= 0.008*) of sage, but at 5 mg/ml there was no change relative to control. The expression of *ced-4* (WS5770) in response to sage was significantly reduced compared to control at each concentration. The downregulation proceeded in a linear pattern with the lowest expression observed at 10 mg/ml (RF=0.583, *p=0.604*), followed by 5 mg/ml (RF=0.604, *p=<0.05*), and 10 mg/ml (RF=0.829, *p=0.001*).

Table 13. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Sage Concentration								
	1 mg/ml			5 mg/ml			10 mg/ml		
	RF	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
tir-1	1.097	0.033	***0.003***	1.203	0.037	***< 0.05***	1.208	0.038	***0.013***
nsy-1	1.364	0.078	***< 0.05***	1.758	0.100	***< 0.05***	1.572	0.265	***0.002***
pmk-1	1.452	0.297	*0.083*	1.718	0.209	***0.006***	1.843	0.402	***0.032***
let-92	3.840	0.520	***<0.05***	3.370	0.669	***0.004***	3.780	0.278	***<0.05***
pptr-1	1.260	0.158	***0.002***	0.970	0.085	***0.001***	1.170	0.047	***<0.05***
nlp-29	0.928	0.158	***0.007***	2.015	1.161	*0.131*	0.619	0.081	***0.017***
nlp-29 D	0.571	0.185	***0.003***	0.558	0.207	*0.065*	0.349	0.071	***0.001***
ced-4	0.829	0.045	***0.001***	0.604	0.018	***<0.05***	0.583	0.017	***<0.05***

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

MAPK Signaling in Response to Sage

Unlike in higher animals, the MAPK signaling cascade in *C. elegans* is mediated by the Toll-interleukin receptor (TIR)-1 cascade (Figure 3). TIR-1 contains a domain that activates signaling implicated in *C. elegans* innate immunity (Couillault et al., 2004). Genetic evidence also suggests that the *C. elegans tir-1*acts downstream of a voltage-gated calcium channel CaKII (UNC-43) to regulate AWC neuron asymmetry (Chuang & Bargmann, 2005). In response to sage extract we observed an increase in expression of *tir-1*. TIR-1 via phosphorylation can activate MAP3 kinase (ASK-1), important in innate immunity which once activated, phosphorylates its downstream target, MAP2 kinase (SEK-1) critical for neuronal identity specification (Chuang & Bargmann, 2005) (Figure 3). ASK-1 (*nsy-1* in *C. elegans*) homolog of can phosphorylate and activate both p38 and

JNK kinases (Chuang & Bargmann, 2005). In response to sage treatment, we observed an upregulation *nsy*-1 and *pmk-1/*p38 MAPK.

However, the expression of antimicrobial neuropeptide *nlp-29* regulated by *pmk-1* cascade decreased in response to sage treatment (Couillault et al., 2004; Pujol et al., 2008; Ziegler et al., 2009). Additionally, we also observed a downregulation in cell-death abnormal-4 (*ced-4*) expression in response to sage treatment. C*ed-4* is conserved from mammalian apoptotic activating factor-1 (Apaf-1) in *C. elegans* that is important in regulation of programmed cell death (Chen et al., 2008; Dasgupta et al., 2007). The member of the caspase family of cysteine proteases and the main executor of apoptosis is caspase 3 or in *C. elegans ced-3*, it is activated into its pro-apoptotic form by *ced-4* (Chen et al., 2008; Pourkarimi et al., 2012).

Even though we noted an increase in the expression of MAP3K and p38 MAPK genes in the MAPK signaling cascade in response to sage treatment, we did not see an increase in the expression of the target genes of the pathway, namely, *nlp-29* and *ced-4*. These results suggest that, although the expression of individual proteins in the cascade increased in response to sage treatment, there was a net decrease in the flux of the pathway, perhaps via inhibition of phosphorylation by phosphatase mediated hydrolysis. These observations are further supported by a multifold increase in the expression of the regulatory phosphatase *let-92*, which is the *C. elegans* PP2A homolog as discussed in chapter-1. Additionally, there was also an increase in the expression of PP2A catalytic subunit *pptr-1* in response to sage treatment suggesting an overall activation of PP2A mediated phosphatase activity.

Conclusion

As discussed above, several lines of evidence have suggested that p38MAPKs play roles in neuronal apoptosis (Cuadrado & Nebreda, 2010) and for protection from exogenous toxins. Furthermore, it has been suggested that coordinated or homeostatic activation of p38 MAPK and JNK is required for axon regeneration (Nix et al., 2011). Other compounds showing promise for treatment of neurodegeneration such as resveratrol have had a biphasic effect on p38 MAPK in a tissue and concentration dependent manner (Schroeter et al., 2002). The regulatory phosphatase PP2A has been shown to inhibit hyperphosphorylation of tau proteins (Liu et al., 2008; Sun et al., 2012). PP2A was shown to regulate apoptosis by dephosphorylating p38 MAPK as well as its apoptotic substrate caspase, playing a pivotal role in reduction of neutrophil driven inflammation (Alvarado-Kristensson & Andersson, 2005). Additionally, p38 MAPK activation was shown to increase transport of the neurotransmitter serotonin in a PP2A dependent process (Zhu et al., 2005). Therefore, homeostatic modulation of the MAPK signaling cascade may be more important than an absolute activation or inhibition of the MAPK cascade as was the case with sage treatments.

CHAPTER IV

TREATMENT WITH SAGE ON HYPOXIA INDUCIBLE FACTOR, APOPTOSIS, AND CELL CYCLE REGULATION IN *C. ELEGANS*

Introduction

Hypoxia Signaling Pathway

Similar to how cells manage oxidative stress via the induction of the Nrf-2/ARE signaling cascade, conditions of hypoxia (O_2 levels of < 21%) are managed at cellular and organismal level by the hypoxia inducible factor-1 (HIF-1) cascade (Dayan et al., 2008). The HIF-1 pathway is made up of a heterodimeric complex of proteins comprising of HIF-1α and HIF-1β (Walmsley et al., 2008). HIF-1β is also termed aryl hydrocarbon receptor nuclear translocator (ARNT) (Ke & Costa, 2006). HIF-1α levels are constantly regulated by the amount of O_2 present in the cell, yet expression is constitutive (Leiser et al., 2011). HIF-1β on the other hand is independent of O_2 levels (Ke & Costa, 2006).

Under the conditions of normoxia, transcriptional activation of the HIF-1 complex is inhibited by a number of different factors. The first one being its interaction with von Hipple-Lindau tumor suppressor protein (VHL), this interaction is facilitated by the hydroxylation of proline residues on HIF-1α by the enzyme proline hydroxylase (PHD) (Kaelin, 2002; Walmsley et al., 2008). This enzyme is 2-oxogluterate iron dependent and

is active under normal oxygen levels (Nagle & Zhou, 2006) (Figure 4). In addition, the enzymefactor inhibiting HIF (FIH) causes hydroxylation of aspartate residues which further prevents the interaction between HIF-1α and HIF-1β, and therefore reduces transactivation (Lando et al., 2002). Upon interaction with VHL this complex is hyperubiquinated and is targeted for proteasomal degradation (Walmsley et al., 2008). Conversely, in conditions of hypoxia where PHDs are inactive the HIF-1 complex dissociates from VHL and becomes transcriptionally active where it translocates in to the nucleus and it binds to the hypoxia responsive element (HRE) region of various target genes (Shen et al., 2005) (Figure 5). This dissociation is further facilitated by phosphorylation of HIF-1α residues by MAPKs which were previously discussed (Chapter 3).

The target genes of the HIF-1 complex are genes that facilitate the response of the cell towards conditions of low O_2 (Walmsley et al., 2008). These include induction of genes that increase the formation of red blood cells, increase O_2 transport by facilitating iron metabolism, and decreasing utilization of O_2 by causing cells to switch to more anaerobic glucose utilization (Nagle & Zhou, 2006). In addition to these metabolic and O_2 dependent changes some of the target genes of HIF-1 are also growth factors. HIF-1 critically regulates and can increase expression of insulin like growth factors (IGF), as well as MAPK and PI3K signaling (Ke & Costa, 2006), all of which causes cellular proliferation and differentiation as described previously (Chapter 1). However, the same is true in regards to cell death. In several cell types HIF-1 is known to activate pro-apoptotic signaling via caspases, Fas and Fas ligand (Ke & Costa, 2006). Moreover, HIF-1 is also known to decrease the activity of the antiapoptotic protein Bcl-2, and upregulate

the pro-apoptotic protein Bcl-2/adenovirus EIB interacting protein 3 (BNip3) (Dayan et al., 2008; Flamant et al., 2010). Thus, a careful balance in cell survival and cell death is known to be regulated by HIF-1 signaling.

Apoptotic Signaling

Apoptotic pathways are induced in cells by a number of different signals, which may either be intrinsic or extrinsic (Perera & Bardeesy, 2011). Extrinsic induction of apoptosis often begins by the binding to receptors of various factors induced in response to oxidative stress or inflammation (Gewies, 2003). These receptors are often referred to as the death receptors, include TNFα-1, and Fas (Kong et al., 2001). Binding of these ligands to the death receptor activates the signaling cascade resulting in the activation of a caspase known as caspase-8 (Gewies, 2003). Once caspase 8 is active it will activate caspase-3, which will in turn activate caspase-6, which results in caspase-7 activation (Gewies, 2003; Lant & Storey, 2010). Under certain circumstances this cascade resulting in the activation of caspase-7 is sufficient to induce apoptosis. Meaning once caspase-7 is active it hydrolyzes many cellular proteins and causes the cell to undergo a classical apoptotic pathway referred to as type I signaling (Gewies, 2003). However, under certain circumstances this type I signaling is not adequate and the apoptotic signaling is then amplified. This amplification is mediated by the mitochondrial BH3-protein Bid which is also activated by caspase-8 (Aggarwal & Shishodia, 2006). Once Bid is active it initiates mitochondrial apoptotic signaling causing the release of cytochrome c from the mitochondria (Kantari & Walczak, 2011). Cytochrome c then forms a complex with apoptotic protease activating factor-1 (Apaf-1) which further activates caspase-3 (Figure 6) increasing the activation of caspases-6 and -7, and therefore increasing the apoptotic

signal (Chai et al., 1999). Mitochondrial apoptotic signaling can also begin intrinsically in type II signaling, independent of death receptor signaling. This can be from mitochondrial damage or oxidative stress, resulting in an increased permeability of the mitochondrial membrane and release of cytochrome c which activates caspase-3 (Wang, 2001). There is also a factor known as the second mitochondrial-derived activator of caspases (Smac) which is another pro-apoptotic factor (Wang, 2001). Under non-apoptotic conditions caspases are inhibited by the inhibitors of apoptosis (IAP) (Caligiuri et al., 2005). When Smacs are released from the mitochondria they prevent the interaction of IAP with caspases allowing caspases to freely become active (Gewies, 2003). IAP expression is controlled by many transcription factors, including HIF-1 (Nadege et al., 2009). All inflammatory transcription factors like NF-kB, TNF-α and TGF-β will upregulate these proteins that can cause inhibition of apoptosis (Glass et al., 2010). There are also other apoptosis inhibitors, namely the before mentioned Bcl-2 that is conserved among several species (Pourkarimi et al., 2012). Bcl-2 will bind to pro-apoptotic proteins such as Bip, Bax, and Bak to prevent apoptosis induction. Bcl-2 also inhibits the activation of caspase-8, the first event in extrinsic induction of apoptosis (Jing et al, 2012).

Regulation of the Cell Cycle

Hypoxia has been shown to play a role in development, which involves several rounds of cell division (Zhang et al., 2009). The division consists of four major phases which are collectively called the cell cycle. The cycle requires duplication of genetic material or DNA synthesis (S phase), and mitotic nuclear division (M phase) (van den Heuvel, 2005). Between these two phases are gap 1 (G_1) and gap 2 (G_2) phases, G_1 lies

between M and S phase while G_2 separates S and M phase (van den Heuvel, 2005). In certain developmental and environmental circumstances the G_1 phase may be arrested, this break in progression is referred to as G_0 (Henley & Dick, 2012). Transition from phase to phase is critically regulated by a group of proteins called cyclin dependent kinases (CDK) (Lant & Storey, 2010). These CDKs require association with a cyclin, or other catalytic subunits for activation (New Science Press, 2007). The expression and degradation of cyclins, and inhibitory proteins is tightly regulated by several transcription factors such as HIF-1 (Ke & Costa, 2006). These inhibitory proteins include the conserved cyclin dependent kinase inhibitors (CDKIs) such as the mammalian p27, which is rate limiting for S phase entry (Ke & Costa, 2006; van den Heuvel, 2005). Furthermore, CDK is also regulated by phosphorylation and dephosphorylation cascades as is HIF-1 (Ke & Costa, 2006; Lant & Storey, 2010).

Sage Treatment for Modification of Hypoxia and Apoptotic Signaling

Natural compounds contained in various plants have been indicated as effective modulators of HIF-1, and cell death or survival pathways (Nagle & Zhou, 2006; Xia et al., 2012). There are both inhibitors and activators of HIF-1, apoptosis and the cell cycle. Although many compounds capable of these modifications have been identified, there is little information on the modulatory effect provided by the *Lamiaceae* herb *Salvia Officinalis* (sage). As previously discussed sage has long been a popular aspect of traditional medicine (Craig, 1999; Naghibi, Mosaddegh, & Motamed, 2005; Yoo et al., 2008), with much of the focus related to its neuroprotecive functions (EL-Kholy et al., 2010; Mohsen Imanshahidi & Hosseinzadeh, 2006). Since sage has been shown to be cytoprotective but very little is known about the affect sage has of hypoxia, apoptosis,

phagocytosis and cell cycle regulation, we explored the interaction between sage extract and key molecular components of these signaling pathways in the nematode *C. elegans*.

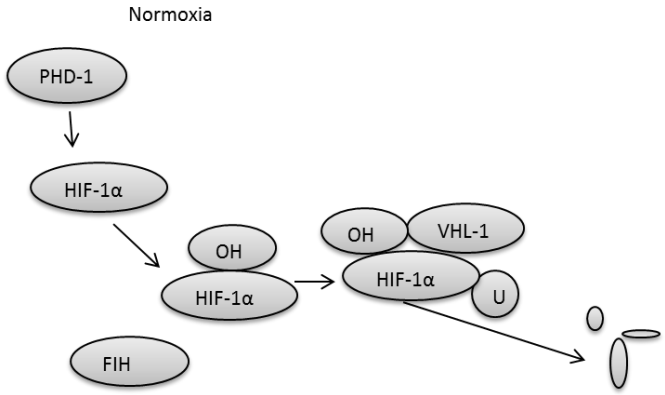

Figure 4. Hypoxia induceable factor-1 (HIF-1) regulation under normal oxygen conditions (normoxia).

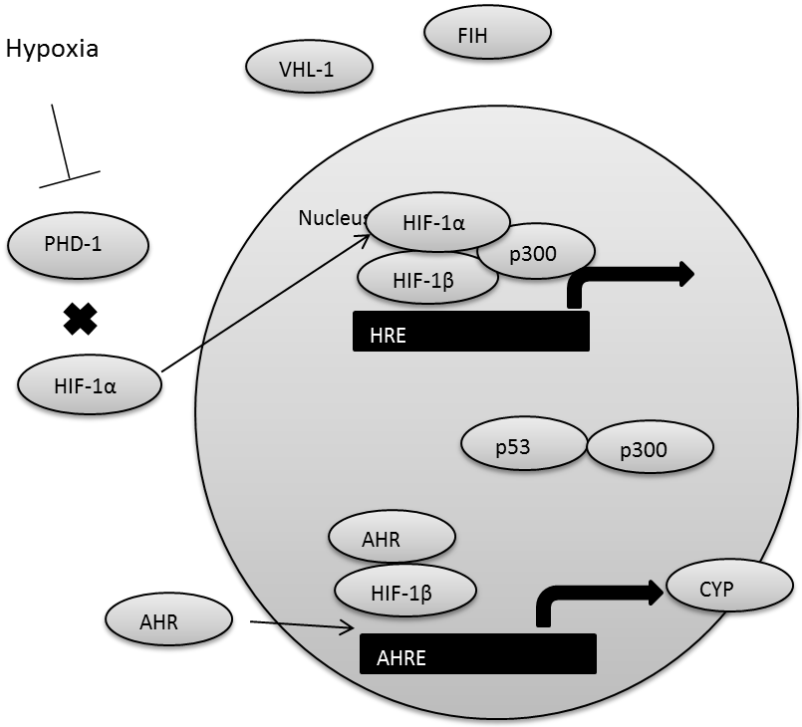

Figure 5. Hypoxia induceable factor-1 (HIF-1) under low oxygen conditions (hypoxia), and the HIF-β competetive binding aryl hydrocarbon receptor (AHR).

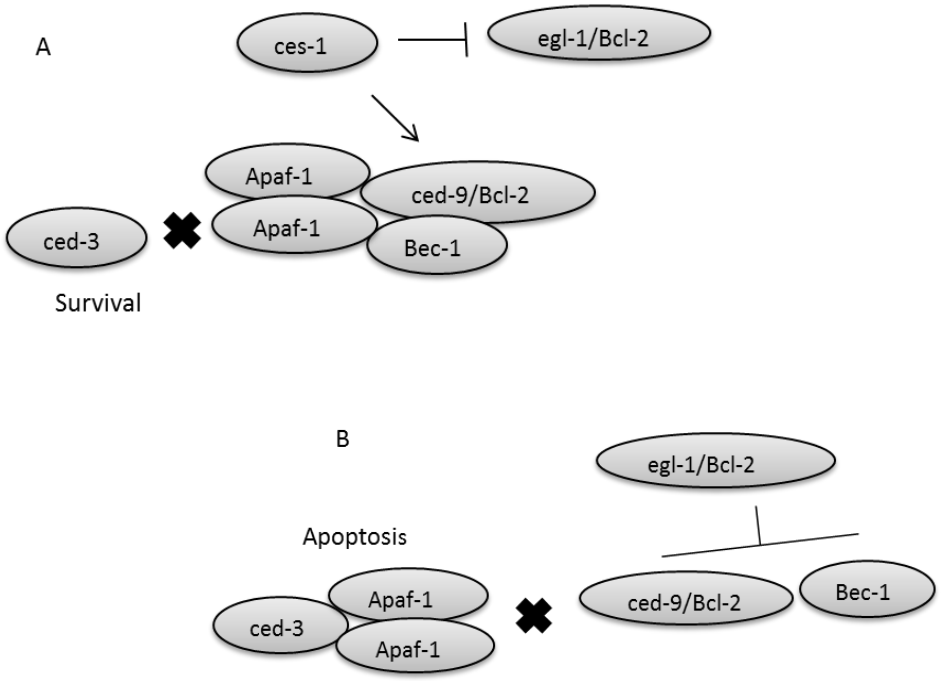

Figure 6. A. Anti-apoptotic signaling resulting in cell survival. B. Pro-apoptotic signaling resulting in cell death. *C. elegans* and human gene names identified.

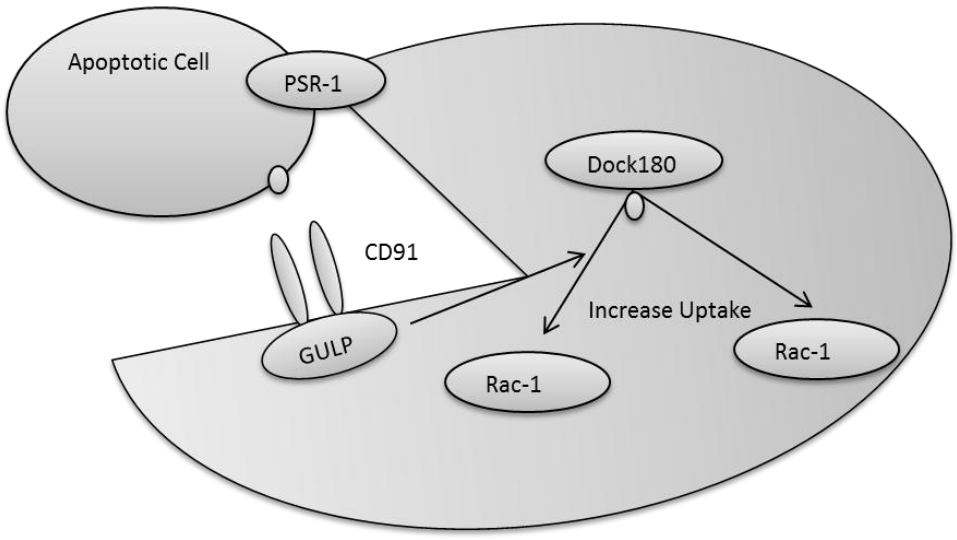

Figure 7. Phagocytic signaling involved in the uptake of apoptotic cells. Human names only

Methods

C. elegans with GFP promoter constructs of genes relevant to HIF-1/Apoptosis/Cell Cycle pathways (Table 14) obtained from the Caenorhabditis Genetics Center were used to study the effect of sage on the expression and activity of genes regulated by HIF-1/Apoptosis/Cell Cycle pathways. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 14. List of *C. elegans* genes in the HIF-1/Apoptosis/Cell Cycle pathways to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Hypoxia Induced Factor/Apoptosis/Cell Cycle			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
OH7688	***hif-1***	Hypoxia-induced factor 1	WBGene00001851
OH7692	***hif-1***	Hypoxia-induced factor 1	WBGene00001851
ZG494	***egl-9***	Prolyl hydroxylase (PHD)	WBGene00001178
BC16164	***fih***	Factor inhibiting HIF	WBGene00017304
UL1606	***aha-1***	Aryl-hydrocarbon receptor nuclear translocator (ARNT) HIF-1β	WBGene00000095
SD1444	***cyp-25a2***	Cytochrome P450 (CYP3A)	WBGene00007964
BC11941	***egl-1***	Bcl-2 homology region 3 mammalian cell death activators	WBGene00001170
BC11395	***bec-1***	Antiapoptitic proteins Beclin1	WBGene00000247
XR-6	***ced-4***	Apoptotic protease activating factor 1	WBGene00000418
WS5770	***ced-4***	Apoptotic protease activating factor 1	WBGene00000418
MD701	***ced-1***	CD91-Low Density Lipoprotein Receptor	WBGene00002989
CU1546	***ced-1***	CD91-Low Density Lipoprotein Receptor	WBGene00000415
NK785	***ced-5***	Protein DOCK180	WBGene00000419
MT10865	***ced-10***	GTPase-Ras-Related C3 Botulinum Toxin substrate (RAC1)	WBGene00000424
VT825	***cki-1***	Cyclin-dependent kinase inhibitor p27/KIP1	WBGene00000516

Results

Effect of Sage on HIF-1/Apoptosis/Cell Cycle

In response to sage treatment expression of hypoxia induced factor 1 (*hif-1*) was downregulated relative to control in two strains of *C. elegans* across all concentrations. The first strain (OH7688) was downregulated the most at 10 mg/ml (RF=0.735, *p=<0.05*), then 5 mg/ml (RF=0.755, *p=<0.05*), followed closely by 1 mg/ml (RF=0.756, *p=<0.05*). The second strain (OH7692) also followed a linear pattern in which the lowest downregulation was seen at 10 mg/ml (RF=0.713, *p=<0.05*), 5 mg/ml (RF=0.722, *p=<0.05*), and 1 mg/ml (RF=0.730, *p=<0.05*). The expression of the gene which codes for the deoxygenase *egl-9* (ZG494) was downregulated in response to sage at 1 mg/ml (RF=0.861, *p<0.05*), 5 mg/ml (RF=0.908, *p=0.005*), and 10 mg/ml (RF=0.831, *p=<0.05*). When compared to control the gene expression of *fih* (BC16164),that codes for 2-oxoglutarate and iron-dependent dioxygenase-related proteins was upregulated in response to sage at 1 mg/ml (RF=1.011, *p=0.019*), downregulated at 10 mg/ml (RF=0.903, *p=<0.05*), and was no different from control at 5 mg/ml. Upregulations in expression for the gene that codes for aryl-hydrocarbon receptor nuclear translocator (*aha-1*) (UL1606) were seen at concentrations of 5 mg/ml (RF=1.190, *p=0.035*), and 10 mg/ml (RF=1.489, *p=0.040*) of sage, but no change in expression was observed at 1 mg/ml. Expression of a gene *cyp-25a2* (SD1444) that codes for a cytochrome P450 was significantly reduced upon sage treatment at 1 mg/ml (RF=0.820, *p=0.020*), and 10 mg/ml (RF=0.790, *p=0.022*), no difference from control was seen at 5 mg/ml.

A linear patter of downregulation was seen when comparing expression with sage treatment to control expression of the gene *egl-1*(BC11941). This gene is homologous to BcL-2 region of mammalian cell death activators, and the lowest decrease was seen at 10 mg/ml (RF=0.544, *p=<0.05*), followed by 5 mg/ml (RF=0.739, *p=<0.05*), and 1 mg/ml (RF=0.823, *p=0.001*). Multidirectional results were seen when evaluating the response in *bec-1* (BC11395) expression to sage treatment, this gene codes for autophagy proteins. At 1 mg/ml (RF=0.705, *p=<0.05*) a downregulation was seen, and at 5 mg/ml there was no significant change over control, yet at 10 mg/ml (RF=1.533, *p=<0.05*) an upregulation was observed. The expression of *ced-4* in response to sage was tested in two strains resulting in overall downregulation. The first strain (XR-6) was upregulated at 1 mg/ml (RF=1.025, *p=0.001*), no different from control at 5 mg/ml, and downregulated at 10 mg/ml (RF=0.868, *p=0.006*). The expression of the second strain (WS5770) was significantly reduced compared to control at each concentration. The downregulation proceeded in a linear pattern with the lowest expression observed at 10 mg/ml (RF=0.583, *p=0.604*), followed by 5 mg/ml (RF=0.604, *p=<0.05*), and 10 mg/ml (RF=0.829, *p=0.001*).

Two *C. elegans* strains were used to test *ced-1* expression, it codes for a low density lipoprotein receptor. The first strain (MD701) was upregulated over control increasing from 1 mg/ml (RF=1.370, *p=<0.05*), to 5 mg/ml (RF=1.874, *p=<0.05*), then 10 mg/ml (RF=1.956, *p=<0.05*). The expression of the second strain (CU1546) was upregulated the highest at 1 mg/ml (RF=1.420, *p=<0.05*), then 10 mg/ml (RF=1.051, *p=0.004*) followed closely by 5 mg/ml (RF=1.037, *p=0.001*). *Ced-5* (NK785) expression was significantly upregulated only at 1 mg/ml (RF=1.489, *p=0.016*), and 10 mg/ml

(RF=1.207, *p=0.012*), 5 mg/ml was no different from control. At each concentration of sage an upregulation of *ced-10* (MT10865) was observed relative to control. The highest increase was at 5 mg/ml (RF=1.999, *p=0.001*), followed by 10 mg/ml (RF=1.625, *p=0.012*), and 1 mg/ml (RF=1.384, *p=<0.05*). The gene (*cki-1)* (VT825) that codes for a cyclin-dependent kinase inhibitor was downregulated upon sage treatment at 5 mg/ml (RF=0.875, *p=0.016*), and 10 mg/ml (RF=0.835, *p=<0.05*), expression at 1 mg/ml was not significantly different than control.

Table 15. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Sage Concentration								
	1 mg/ml			5 mg/ml			10 mg/ml		
	RF	SE	*p value*	RF	SE	*p value*	RF	SE	*p value*
hif-1	0.756	0.031	***<0.05***	0.755	0.040	***<0.05***	0.735	0.025	***<0.05***
hif-1	0.730	0.027	***<0.05***	0.722	0.035	***0.001***	0.713	0.038	***<0.05***
egl-9	0.861	0.024	***<0.05***	0.908	0.038	***0.005***	0.831	0.027	***<0.05***
fih	1.011	0.053	***0.019***	1.061	0.048	*0.300*	0.903	0.059	***<0.05***
aha-1	0.998	0.279	*0.495*	1.190	0.126	***0.035***	1.489	0.280	***0.040***
cyp-25a2	0.820	0.037	***0.020***	0.905	0.057	*0.281*	0.790	0.051	***0.022***
egl-1	0.823	0.050	***0.001***	0.739	0.026	***<0.05***	0.544	0.020	***<0.05***
bec-1	0.705	0.042	***<0.05***	1.097	0.114	*0.298*	1.533	0.100	***<0.05***
ced-4	1.025	0.029	***0.001***	0.959	0.063	*0.439*	0.868	0.049	***0.006***
ced-4	0.829	0.045	***0.001***	0.604	0.018	***<0.05***	0.583	0.017	***<0.05***
ced-1	1.370	0.057	***<0.05***	1.874	0.102	***<0.05***	1.956	0.138	***<0.05***
ced-1	1.420	0.085	***<0.05***	1.037	0.033	***0.001***	1.051	0.042	***0.004***
ced-5	1.489	0.231	***0.016***	1.148	0.119	*0.086*	1.207	0.133	***0.012***
ced-10	1.384	0.102	***<0.05***	1.999	0.288	***0.001***	1.625	0.258	***0.012***
cki-1	0.956	0.085	*0.200*	0.875	0.070	***0.016***	0.835	0.051	***<0.05***

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

HIF-1/Apoptosis/Cell Cycle Signaling in Response to Sage

Optimal management of O_2 levels at both cellular and organismal level is critical for overall health of the system. As discussed previously conditions of hyperoxia are managed by Nrf2/ARE (Godman et al., 2010), whereas conditions of hypoxia are now understood to be managed by HIF-1 signaling (A. J. Chang & Bargmann, 2008). However, the homeostatic achievement of normoxia is a result of a careful balance between Nrf2 and HIF-1 signaling. It is quite important that any type of dietary treatment does not result in over expression or activation of either of these two signaling pathways, especially since an overactive HIF-1 signaling has been implicated in unregulated cell growth resulting in cancer (Shen et al., 2005). In our studies we evaluated the effects of sage on various critical factors involved in HIF-1 signaling. As mentioned when O_2 levels are high the HIF-1α subunit is hydroxylated and targeted for degradation by the VHL-1 tumor suppressor protein (Shen et al., 2005). This regulatory pathway is evolutionarily conserved, and the human HIF-1α subunit is equivalent to *hif-1* in *C. elegans* (Shen et al., 2005). Our results indicated that treatment with sage resulted in an overall downregulation in expression of the *hif-1*/HIF-1α subunit suggesting that treatment did not result in hypoxia conditions since we know that under hypoxic conditions HIF-1α is stabilized. Similarly, the heavily investigated green tea compound (-)-epigallocatechin-3-gallate (EGCG) inhibited HIF-1α protein accumulation, and reduced protein and mRNA levels of HRE downstream growth factors in cell culture (Q. Zhang et al., 2006).

In *C. elegans* HIF-1β or ARNT is *aha-1*, which as mentioned earlier interacts with *hif-1* for binding to the HRE (Zhang et al., 2009). The expression of *aha-1*/HIF-1β was found to be upregulated in response to treatment with sage. It is already known that HIF-1β levels are unaffected by O_2 concentrations in the cell, however *aha-1*/HIF-1β can form a heteromeric dimer with aryl hydrocarbon receptor (AHR) and bind to the aryl hydrocarbon responsive element (AHRE) region of various genes (Denison & Nagy, 2003). It is possible that if sage bioactive compounds are metabolized by AHR/HIF-β/AHRE signaling we would see an upregulation in genes that are controlled by AHRE. AHR/ARNT target genes encode phase I enzymes such as cytochrome P450 (CYP 450) and phase II xenobiotic-metabolizing enzymes like glutathione-S transferase (GST) (Nebert et al., 2004). However, we saw that the gene expression of one such cytochrome CYP3A (*cyp-25a2* in *C. elegans*) was either downregulated or did not change in response to sage treatment. This suggests two things, first sage compounds were not activating AHR signaling, and second it also suggests that the excess *aha-1*/HIF-1β was not forming heterodimers with AHR and becoming transcriptionally active, perhaps they were being targeted for proteasomal degradation.

FIH (*fih-1* in *C. elegans*) inactivates *hif-1*/HIF-1α under normal oxygen levels (Lando et al., 2002). We saw an increase in the expression of *fih-1*/FIH-1 at lower concentration but a significant downregulation observed at the highest concentration. As discussed *fih-1* hydroxylates the aspartate residues and reduces the interaction between HIF-1α and HIF-1β, thereby allowing accumulation of *aha-1*/HIF-1β. Similarly, the cytoprotective compound quercetin was shown to inhibit FIH only at high doses (Nagle & Zhou, 2006). Excessive *aha-1*/HIF-1β is perhaps engulfed by lysosomes and degraded.

Interestingly, we also saw a downregulation in PHD-1 (*egl*-9 in *C. elegans*) which suggests that conditions of normoxia were maintained in the cell. We know that *egl-9*/PHD-1 uses O_2 as a substrate to carry out hydroxylation of proline residues, and its levels typically only go down in hypoxia (Shao et al., 2009). This suggests that a possible achievement of normoxia at the cellular as well as organismic level in *C. elegans* in response to sage treatment.

Studies in *C. elegans* have revealed a conserved apoptotic pathway (Hirose et al., 2010). This pathway consists of the genes such as egg-laying defective-1 (*egl-1*), three cell-death abnormal (ced), *ced-9, ced-4,* and *ced-3*, and the transcription factor cell-death specification-1 (*ces-1* in *C. elegans*) which can directly repress expression of the BH3-proapoptotic gene *egl-1/*Bcl-2 and prevent the death of motor neuronal cells (Hirose et al., 2010). Caspase-3 is the main executor of apoptosis *(ced-3* in *C. elegans*), which is activated to the pro-apoptotic form by *ced-4*/Apaf-1 as discussed in chapter 3 (Chen et al., 2008). Since our treatment with sage resulted in a downregulation of the HIF-1 response we wanted to evaluate if it resulted in a concomitant increase in apoptotic signaling. As HIF-1 is known to promote cell growth and differentiation, inhibition of HIF-1 signaling should result in an increase in apoptotic signaling. In response to sage treatment the proapototic protein *egl-1/*Bcl-2 was downregulated. *Egl-1*/Bcl-2 decreases the interaction of the BH3-antiapototic *ced-9/*Bcl-2 protein on the mitochondrial membrane with the additional antiapototic protein *bec-1*/Bec-1 and *ced-4*/Apaf-1 dimers (Chen et al., 2008) (Figure 6). When proapoptotic *egl-1*/Bcl-2 binds to antiapoptotic *ced-9/*Bcl-2 it releases the *ced-4/*Apaf-1 dimer which is then free to activate pro-caspase-3 into caspase-3 (*ced-3* in *C. elegans*) (Figure 6). *Ced-3*/Caspase-3 once active will

increase various apoptotic cascades in the cell. Therefore downregulation in *egl-1/*Bcl-2 expression suggests that *ced-3*/caspase-3 mediated apoptotic signaling was not actively induced by sage. Similarly, the *Lamiaceae* herb *Rosmarinus Officinalis* (rosemary) was shown to be neuroprotective against ROS-induced apoptosis in human dopaminergic neurons by suppressing Bax, Bak, caspase-3, and down-regulating proapototic Bcl-2. Furthermore, an overall increase in the expression in antiapoptotic *bec-1/*Bec-1, and also a decrease in *ced-4/*Apaf-1 dimers suggests that there is a lower amount of *ced-4/*Apaf-1 bound to *ced-9/*Bcl-2 or free in the cell for lowered activation of *ced-3*/caspase-3 (Takacs-Vellai et al., 2005). Therefore it can be concluded that sage treatment did not result in an increase in apoptotic signaling even though HIF-1 was decreased.

We also wanted to see if sage treatment resulted in any changes in nutrient uptake or nutrient recycling carried out via phagocytosis. Therefore, we looked at the expression of various proteins in *C. elegans* that either recognize an apoptotic cell or are involved in cellular uptake of apoptotic cells. It has been shown that the transmembrane low density lipoprotein receptor molecule CD91 (*ced-1 in C. elegans*) recognizes an extracellular protein on apoptotic cells (Moreira & Barcinski, 2004; Wang et al., 2011). This receptor interacts with GULP (*ced-6* in *C. elegans*), which is co-localized with *ced-1*/Apaf-1 at the plasma membrane and around engulfed apoptotic cells (Xiaochen Wang et al., 2011) (Figure 7). *Ced-1*/CD91 expression in response to sage treatment was upregulated overall. Also of importance are the proteins, *ced-5* (human Dock180) and *ced-10* (human Rac-1) which are involved in increasing the efficiency of phagocytosis (Moreira & Barcinski, 2004) (Figure 7). The results indicated that sage treatment resulted in an overall upregulation of these phagocytic proteins. Suggesting that, there is an increase in

the efficiency of nutrient utilization in response to sage treatment (Stuart & Ezekowitz, 2005). This also suggests that treatment with sage resulted in active removal of damaged cells, suggesting that the treatment resulted in overall reduction in the accumulation of oxidized proteins, nucleic acids, and lipids which might be a direct result of the ability of sage extracts to reduce oxidative stress, also reduce the accumulation of defects resulting from oxidative stress (Sayre et al., 2008). It is well known that sage has antioxidant, and anti-mutagenic activities. Our findings may suggest one of the mechanisms by which sage extracts mediate their antioxidant and anti-mutagenic effects (Keshavarz et al., 2010; Xavier et al., 2009), not by physically quenching the oxidative species but by facilitating active removal of age or oxidation damaged cells.

Furthermore, we also investigated the effect of sage on CKDI (*cki-1* in *C. elegans*) which inhibits primarily cyclin D (*cdy-1*) mediated cell cycle signaling. Cyclin D activation requires ubiquitin proteosomal degradation of CKI, CDK phosphorylation by activating kinases, and removal of inhibitory phosphates (van den Heuvel, 2005). As mentioned c*ki-1* is a negative regulator of cell cycle entry and is analogous to p27 in mammals (van den Heuvel, 2005). Our study indicated a slight downregulation in expression of *cki-1*, but only at high concentrations, at the lowest concentration there was no significant difference. This further suggests that overall modulatory effect of sage on various aspects of cell growth, differentiation, and apoptosis. Since cyclin D is involved in active progression of cells from G_1 to s phase where it functions in association with cyclin-dependent kinase 4 (*cdk-4*), transcription factor E2F (*efl-1*), and cyclin E (*cye-1*) (Tilmann & Kimble, 2005). *Cki-1* actively inhibits cyclin D and reduces the transition of cells from G_1 to S phase. This transition is extremely important in developmental

progression of *C. elegans*, specifically important in differentiation of cells into gonadal as well as neuronal cells (Chang et al., 2004). Our results indicate that there was no negative effect on developmental progression, and in fact at higher concentrations sage may actually increase gonadal and neuronal development in *C. elegans*.

Conclusion

The overall effect of sage on four important signaling pathways HIF-1, apoptosis, phagocytosis, and cell cycle regulation was evaluated. Our results indicate that there was a marginal decrease in HIF-1 signaling in response to sage treatment. However, this decrease in HIF-1 did not result in a concomitant increase in apoptotic signaling, but there was an increase in expression of genes related to phagocytosis. Suggesting there was active and efficient nutrient utilization or an enhanced ability to clear apoptotic cells. We also did not notice any negative effects on cyclin D mediated cellular development and differentiation with sage treatment, at higher levels there may be an increase in differentiation of cells into gonadal and neuronal cells suggesting a possible beneficial effect of sage on these tissues.

CHAPTER V

THE *IN VIVO* EFFECT OF SAGE EXTRACT ON THE HSP AND UPR PATHWAYS

Introduction

Hsp/UPR Signaling

The heat-shock protein (Hsp) and unfolded protein response (UPR) signaling are mechanisms by which cells regulate a stress response, and carefully monitor the protein quality at a cellular level (Cyr & Hebert, 2009; Haynes & Ron, 2010). The protein quality in eukaryotes is affected by the amount of protein synthesized by the endoplasmic reticulum (ER), and by the amount of protein taken up into the cell as a result of apoptosis and phagocytosis (Matus et al., 2008). It is important that cells very quickly get rid of the proteins that are not correctly folded or damaged. Misfolded proteins can aggregate resulting in their precipitation which can initiate apoptotic signaling (Arya et al., 2007).

In eukaryotes the amount of misfolded protein and its quality control are regulated by 4 distinct pathways (Cyr & Hebert, 2009). The most important of which is the pathway regulated by the heat-shock family (HSF) of transcription factors (Haynes & Ron, 2010). Under normal circumstances when the levels of misfolded proteins are low HSF-1 is bound by its chaperone protein, most commonly Hsp70 (Wieten et al., 2010).

However, when the levels of unfolded proteins accumulate in the cytoplasm it causes the dissociation of Hsp70 from HSF-1, and Hsp70 binding to the misfolded proteins leaves free HSF-1 (Wieten et al., 2010) (Figure 8). When phosphorylated HSF-1 undergoes trimerization, and it becomes transcriptionally active and translocates into the nucleus (Haynes & Ron, 2010). There in reaction to an additional signal it can bind to promoter regions of various genes that recognize the HSF-1 response element and transcribes genes that further facilitate the handling of misfolded proteins in cells, typically results in expression of more Hsps (Haynes & Ron, 2010). In addition to the amount of misfolded proteins the activity of HSF-1 is also independently regulated by several other signaling cascades such as insulin like growth factor (IGF) signaling (Prahlad & Morimoto, 2008). As described in the first chapter IGF is important for regulation of oxidative stress as well as growth and development. When IGF signaling is active it can actively phosphorylate HFS-1 causing it to dissociate from Hsp70 facilitating its activation for gene expression (Prahlad & Morimoto, 2008). Other types of growth factor related signaling responses are also known to activate HSF-1mediated signaling cascades (Baumeister et al, 2006).

A second pathway by which cells regulate the level of misfolded proteins entering or exiting the cell is via the and protein kinase RNA-like endoplasmic reticulum kinase (PERK) cascade (Fels & Koumenis, 2006). This protein is typically membrane bound, when it is active it phosphorylates the eukaryotic transcription factor elf2α, which when it is phosphorylated it reduces no-chaperone transcription thereby reducing the burden on the ER (Cyr & Hebert, 2009; Fels & Koumenis, 2006) (Figure 8).

In the presence of misfolded proteins there is also activation of a third signaling pathway which is inositol-requiring enzyme 1 (IRE1) mediated. IRE is typically bound to

the cell membrane by a chaperone, when activated it is released into the cytoplasm where it activates an endonuclease XLP21, which splices the genes for XBP1 (Haynes & Ron, 2010) (Figure 8). This XBP1 is then transcribed to make the XBP1 protein which itself is a transcription factor, and once it is active it regulates genes involved with protein stress (Cox et al., 2011).

The fourth pathway is similar to IRE, it is mediated by ATF6 which like IRE is also bound to the ER (Matus et al., 2011). Upon increase in cytoplasmic unfolded protein levels it is released into the cytoplasm. ATF6, once released also acts as a transcription factor for the expression of several genes that facilitate handling of misfolded proteins like immunoglobulin binding protein (BiP) (Gavilán et al., 2009) (Figure 8).

Regulation of Apoptotic Signaling

One of the main outcomes of initiation of Hsp signaling in addition to increasing the ability of cells to handle the amount of unfolded proteins to increase the survival of the cells is by the initiation of anti-apoptotic genes such as bcl-2 (Davenport et al., 2007). However, when the levels of misfolded proteins increases beyond the capacity of the signaling cascade to handle then it activates an apoptotic signal mediated by CHOP via PERK and ATF6 induction, resulting in suppression of antiapoptotic bcl-2 transcription and induction of proapoptotic and cell-cycle arresting genes (Davenport et al., 2007). Additionally, activation of the intrinsic apoptotic pathway results in the direct activation of caspases as well as release of cytochrome c from the mitochondria, release of Apaf1, and subsequent activation of caspase-3 as discussed previously (Chapter 4) (Fels & Koumenis, 2006; Matus et al., 2011). Because of unification of oxidative stress response

signaling with the Hsp signaling these two pathways usually work in tandem to ensure a careful banance between cell survival and cell death (Calabrese et al., 2008). This balance is regulated via redox status but also the amount of protein stress due to not only misfolded proteins but also proteins from microbial infection since these are taken up by the cell and recognized as foreign or misfiled (Glass et al., 2010). Therefore there is a careful integration between these protein stress response pathways which can ultimately decide the fate of the cell.

Hsp/UPR Link to Neurodegeneration

Proper response by these systems is vital since when misfolded proteins aggregate they form insoluble intracellular or extracellular deposits, which are toxic and lead to apoptosis (Jing et al., 2012). It has been demonstrated that age and inflammation related neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are associated with the build-up of misfolded or unfolded protein aggregates (Jing et al., 2012; G.-rui Luo, Chen, & Le, 2007). In AD Amyloid $\beta_{1\text{-}42}$ miss assembly and accumulation is increased upon the lowering of HSF-1 levels (Magrané et al., 2005). Several sHsps such as *hsp16.2* have been shown to be components of AD linked amyloid $\beta_{1\text{-}42}$ peptide aggregatory plaques (Fonte et al., 2008). Although there are varying opinions these sHsps are generally considered to be a protective response and not contribute to the pathology (Fonte et al., 2008; Kirbach & Golenhofen, 2011). Additionally, it has been shown that increase in Hsp70 is also important for PD since elevated levels can bind to α-synuclein and prevent accumulation and thus prevent the formation of neurotoxic neurofribrillary tangles known as Lewy bodies (Arawaka et al., 2010; Malkus et al., 2009).

Individual neurodegenerative diseases manifest in different neuronal cell types, however oxidative stress, misfolded proteins, and suppression of neuronal signaling are common pathological features. Currently neurodegenerative diseases lack effective treatment options, many available therapies do no more than treat the symptoms (Kelsey et al., 2010).

Sage on Neurodegenerative Conditions/ Protein Homeostasis

As previously discussed the *Lamiaceae* herb *Salvia Officinalis* (sage) contains numerous phytochemicals that have displayed various neurological benefits including the prevention of peptide and protein accumulation relevant to AD and PD (Iuvone et al., 2006; Sul et al., 2009). In humans sage treatment has improved cognitive function in patients with moderate AD, as well as improved mood and cognitive performance in healthy individuals (Iuvone et al., 2006; Kennedy et al., 2006, 2011; Perry et al., 2003; Scholey et al., 2008). Additionally, sage has been shown to increase Hsp70 expression in humans (Sá et al., 2009). Provided this evidence on the possible protective actions of sage and the general consensus that protein folding plays a role in neurodegenerative disease we evaluated the response in expression to sage in key genes in both the Hsp and UPR signaling pathways using the nematode *C. elegans*.

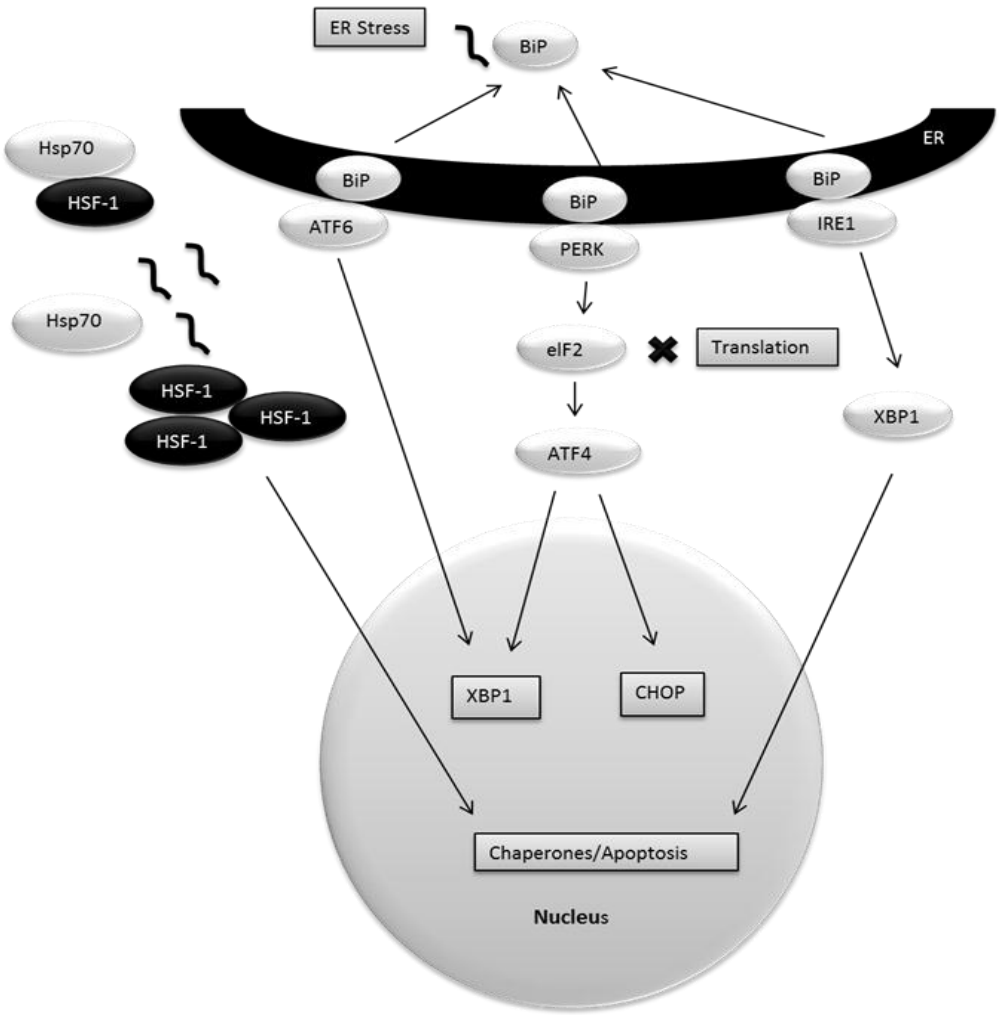

Figure 8. The heat shock protein response (Hsp) and the unfolded protein response (UPR) in management of protein stress.

Methods

C. elegans with GFP promoter constructs of genes relevant to Hsp/UPR pathways (Table 16) obtained from the Caenorhabditis Genetics Center were used to study the effect of sage on the expression and activity of genes regulated by Hsp/UPR pathways. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 16. List of *C. elegans* genes in the Hsp/UPR pathway to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Heat Shock Protein/ Unfolded Protein Response			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
CL2070	***hsp16.2 D***	16-kD heat shock protein; αB crystalline driven (D)	WBGene00002016
GS1826	***phsp***	Driven by heat shock protein-16.2,and heat shock protein-16.41; αB crystalline	WBGene00002016; WBGene00002018
SJ6	***hsp-4***	Immunoglobulin binding protein (BiP)	WBGene00002008
BC13292	***dyn-1***	Dynamin GTPase	WBGene00001130
SJ4063	***abu-1***	Activated in Blocked Unfolded Protein Response	WBGene00000024
DH1336	***rme-8***	Receptor Mediated Endocytosis	WBGene00004378

Results

Effect of Sage on Hsp/UPR Signaling

The gene expression in response to sage extract was evaluated relative to control expression at concentrations of 1 mg/ml, 5 mg/ml, and 10 mg/ml. The first *C. elegans* strain (CL2070) evaluated codes genes driven by *hsp16.2*. We observed downregulations at 1 mg/ml (RF=0.695, $p= 0.019$), and 5 mg/ml (RF=1.831, $p= 0.046$), while upregulation was seen at 10 mg/ml (RF=1.831, $p=0.041$). The *C. elegans* gene coded by *phsp* (GS1826) is induced by heat and is driven by *hsp16.2*, and *hsp16.4*. This gene was upregulated at 1 mg/ml (RF=1.507, $p=0.010$), and 10 mg/ml (RF=1.283, $p=0.036$). When compared to control, expression of *hsp-4* (SJ6) was downregulated at 1 mg/ml (RF=0.848, $p=<0.05$), and 5 mg/ml (RF=0.985, $p=0.003$), but expression was elevated at 10 mg/ml (RF= 1.139, $p=0.045$).

UPR was evaluated using three *C. elegans* genes, the first of which was *dyn-1* (BC13292). We observed an upregulation in expression at 1mg/ml (RF=1.242, *p=0.005*), however the response to sage was not statistically different from control at 5 mg/ml and 10 mg/ml. We then tested the gene *abu-1* (SJ4063), and in response to sage the expression was upregulated from control at 1 mg/ml, (RF=1.034, *p=0.002*), and downregulation occurred at 5 mg/ml (RF=0.857, *p=0.020*), and 10 mg/ml (RF=0.886, p=0.016). The expression of *C. elegans rme-8* (DH1366) was significantly downregulated from control at 1 mg/ml (RF= 0.951, *p=0.042*), expression in response to sage was upregulated at 5 mg/ml (RF=1.037, *p=0.004*) and at 10 mg/ml was no different from control.

Table 17. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Sage Concentration								
	1mg/ml			5mg/ml			10mg/ml		
	RF	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
hsp16.2 D	0.695	0.013	***0.019***	0.792	0.018	***0.046***	1.831	0.377	***0.014***
phsp	1.507	0.190	***0.010***	1.550	0.369	*0.092*	1.283	0.157	***0.036***
hsp-4	0.848	0.150	***<0.05***	0.985	0.189	***0.003***	1.139	0.165	***0.045***
dyn-1	1.242	0.122	***0.005***	1.158	0.033	*0.065*	0.869	0.054	*0.481*
abu-1	1.034	0.076	***0.002***	0.857	0.076	***0.020***	0.886	0.049	***0.016***
rme-8	0.951	0.030	***0.042***	1.037	0.038	***0.004***	1.267	0.191	***0.0513***

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

Hsp/UPR Signaling in Response to Sage

We evaluated the expression of *C. elegans* genes under control of the *hsp16.2* promoter these Hsps are small inducible proteins homologous to vertebrate αB crystalline

(Fonte et al., 2008). Previous *C. elegans* studies have indicated that *hsp16.2* is protective since it is upregulated in response to consumption of exogenous compounds, such as the pro-oxidant chemical juglone (Hartwig et al., 2009). It has also been shown that over expression of *hsp16.2* suppressed amyloid-β toxicity in a *C. elegans* AD model (Fonte et al., 2008). We saw downregulations of this protein at the lowest concentrations, but at the highest concentration there was an upregualtion. We also evaluated a *C. elegans* stain expressing the transgene *phsp*, this promoter region is shared by heat inducible *hsp16.2* and *hsp16.41* (Hong et al., 2004). In response to sage treatments expression was upregulated at the lowest and highest concentrations. This indicates an overall increased expression of the small hsp16.2 can be achieved upon sage treatment.

We also studied the UPR in response to sage treatments. We observed an increase in the expression of *C. elegans hsp-4*, the mammalian chaperone protein BiP. The expression of *hsp-4* was concentration dependent, at low levels we saw a decrease yet at high levels there was an increase. This indicates that the *hsp-4*/BiP levels are changing in response to sage, and since it is a broad spectrum chaperone once its expression levels increase it will bind to many misfolded proteins in the cytoplasm conferring protection against protein stress (Jo et al., 2009). Increasing levels of expression means that there may be increased cellular protection. However, this also means that there is an increased amount of misfolded proteins present upon treatment with higher concentrations of sage. The decreased expression at lower concentrations of sage indicates that there is less *hsp-4*/BiP to bind to ATF6 and IRE. This would free ATF6 and IRE allowing them to be free and thus transcriptionally active. As discussed previously when these genes become transcriptionally active they induce cell survival (Cox et al., 2011; Jo et al., 2009; Urano

et al., 2002) (Figure 8). Since there is less amount of misfolded protein entering and simultaneously there is an increase of cell survival signaling it can be hypothesized that at low levels sage can promote cell survival in neuronal cells (Jo et al., 2009; Matus et al., 2008; Prahlad & Morimoto, 2008).

In addition to UPR mediated ER stress there is an additional pathway mediated by activated in blocked UPR-1 (*abu-1* in *C. elegans*). In the conditions of impaired UPR signaling *abu-1* helps cells regulate protein stress (Song et al., 2010). We saw an upregulation in *abu-1* at the lowest concentration of sage, but downregulations at the higher concentrations. Suggesting that this was the either the primary or an additional mechanism of protection against protein stress at lower concentrations of sage. At higher levels *abu-1* was downregulated suggesting that UPR then becomes the more dominant method of handling protein stress.

Dynamin (*dyn-1*) has been shown to function in *C. elegans* endocytosis, a process necessary to internalize fluid from the extracellular medium, intake nutrients, and membrane component recycling. The large GTPase dynamin is involved in pinching off the clatherin coated pit from the plasma vesicle (Song et al., 2010). In response to sage treatment we observed upregulation in *dyn-1* expression at the lowest concentration, but no significant difference in expression at higher concentrations. This suggests that there was quick uptake and transport of misfolded or foreign proteins, thus the efficiency of protein delivery was increased which possibly contributes to overall improved handling of misfolded proteins in the cell. There was also an increase in recruitment of Hsp70 chaperone proteins to the cell membrane by their co-chaperone binding partner known as receptor-mediated endocytosis (RME)-8 or *rme-8* in *C. elegans* (Girard et al., 2005).

Conclusion

The effect of sage on protein stress in *C. elegans* was concentration dependent, at lower concentrations it resulted in both UPR and *abu-1* mediated signaling, whereas at high concentrations it resulted in only increased UPR signaling. However, the *hsp16.2* increased in general in response to sage treatment. Even the overall increased colocalization of Hsp70 with *rme-8* suggests that sage treatment provides improved handling of misfolded or foreign proteins by cells and might result in increased cell survival by modulation of Hsp/UPR signaling in combination with the modulation of insuling/IGF signaling previously discussed (Chapter 1).

CHAPTER VI

THE *IN VIVO* MODULATION OF TGF-β SIGNALING IN RESPONSE TO SAGE TREATMENTS

Introduction

TGF-β Signaling Pathway

The transforming growth factor β (TGF-β) signaling pathway is involved in both proliferation and cell differentiation (Lant & Storey, 2010). It responds to signaling in neurons, and reactive to external signals including environmental cues (Lant & Storey, 2010). The TGF-β pathway has parallels with the insulin/insulin like growth factor (IGF) pathway and its expression is also typically seen under conditions conducive to growth that involve energy expenditure (Prahlad & Morimoto, 2008).

The TGF-β family is a diverse group of growth factors and developmental elements, which includes ligands, as well as bone morphogenic proteins (BMPs) (Lant & Storey, 2010). There are two kinase receptors TGF-β type I and II, these receptors form a heteromer when a ligand is bound (Savage-Dunn, 2004). Overall pathway of signaling involves ligand binding at the cellular TGF- β I receptor then phosphorylation and activation of TGF- β I receptor by TGF-β II, followed by downstream messaging via activation of a family of transcription factors known as mothers against decapentalegic

(Smad) (Lant& Storey, 2010). These Smads form a complex that binds to the Smad-binding elements in the promoter regions of genes (Suenaga et al., 2008). Additionally, TGF-β receptors can activate MAPKs such as p38, JNK, and function in parallel to PI_3K/AKT signaling which is not related to Smad signaling (Kang et al., 2009).

Neurodegenerative Disease and TGF-β Signaling

The TGF-β receptor has a controversial role in the pathogenesis of neurodegenerative disease (Lee et al., 2010). Reduced levels of TGF-βII are observed early in animal and human Alzheimer's disease (AD) brains (Lee et al., 2010). Research has also linked AD pathology to reduction of TGF-βI receptors, independent of the type II receptor (Caraci et al., 2011; Caraci et al., 2012). However, overexpressing TGF-βI receptors also leads to neuronal apoptosis, and has been associated with deposition of extracellular matrix, which reduces clearance of neurotoxic amyloid β in AD (Lee et al., 2010). Therefore, homeostatic regulation of TGF-β signaling may be critical in prevention or management of neurodegernative disease.

Modulation of TGF-β Signaling with Sage

Numerous studies have shown the herb *Salvia Officinalis* (sage) to be neuroprotective (EL-Kholy et al., 2010; Kennedy et al., 2006; Keshavarz et al., 2010; Perry et al., 2003; Scholey et al., 2008). Sage contains many compounds that have been shown to modulate stress response signaling pathways including TGF-β and insulin signaling (Ingh et al., 2008; Kapiszewska et al., 2005; Kennedy & Wightman, 2011; K. H. Kim et al., 2011). Therefore, we evaluated the expression of genes relevant to TGF-β signaling in response to sage treatments using a transgenic *C. elegans* model.

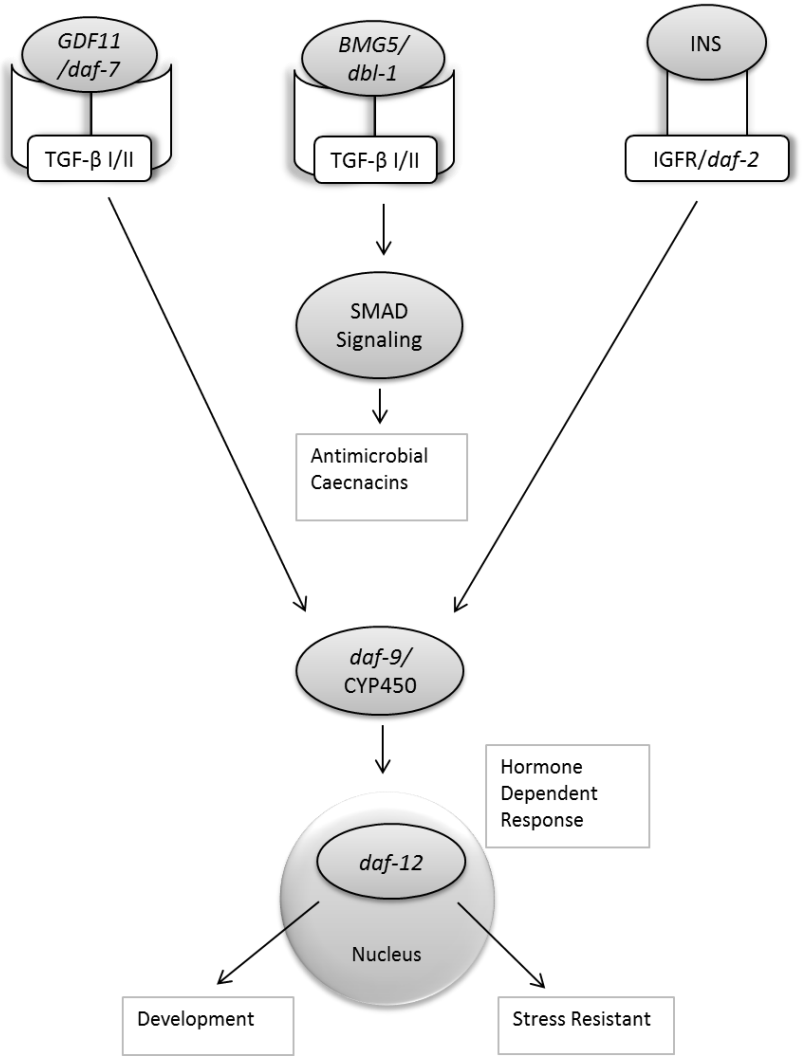

Figure 9. Transforming growth factor β (TGF-β) signaling in parallel with insulin/insulin growth factor (IGF) signaling. Human and *C. elegans* identifiers.

Methods

C. elegans with GFP promoter constructs of genes relevant to the TGF-β signaling pathway (Table 18) obtained from the Caenorhabditis Genetics Center were used to study the effect of sage on the expression and activity of genes regulated by TGF-β system. *C. elegans* treatment propagation and quantification of gene expression were done using methods described above in chapter 1.

Table 18. List of *C. elegans* genes in TGF-β pathway to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Transforming Growth Factor Beta			
Strain	**Gene**	**Human Homolog**	**Wormbase-Gene ID**
BW1940	***dbl-1***	Transforming growth factor β-bone morphogenetic protein 5 (BMP5)	WBGene00000936
AA278	***daf-9***	Cytochrome P450 (CYP2 subfamily) steroidogenic or fatty acid hydroxylase	WBGene00000905
AA120	***daf-12***	Nuclear receptor (Vitamin D)	WBGene00000908
FK181	***daf-7***	Transforming growth factor β-related proteins, Growth differentiation factor 11 (GDF11) (BMG-family)	WBGene00000903
BC14074	***daf-2***	Insulin/Insulin growth factor (IGF) receptor	WBGene00000898

Results

Effect of Sage on TGF-β Signaling

The expression of *dbl-1* (BW1940) was upregulated at 5 mg/ml (RF=1.312, $p=< 0.05$), and 10 mg/ml (RF=1.436, $p= 0.029$) of sage but was no different from control at 1 mg/ml (Table 19). In response to sage expression of a *C. elegans* gene *daf-9* (AA278) that codes for a cytochrome P450 was significantly upregulated at each test concentration. The highest upregulation was seen at 10 mg/ml (RF=1.520, $p=< 0.05$), followed by 1 mg/ml (RF=1.467, $p=<0.05$), and 5 mg/ml (RF=1.378, $p=< 0.05$) (Table 19.). Multifold upregulations of *daf-12* (AA120), the gene that codes for a vitamin D receptor were seen in response to sage at 1 mg/ml (RF=3.751, $p= < 0.05$), 5 mg/ml (RF= 3.757, $p=< 0.05$), and 10 mg/ml (RF=3.746, $p= < 0.05$) (Table 19). Significant

downregulations in *daf-7* (FK181) expression were seen in response to sage at 1 mg/ml (RF= 0.880, *p= 0.002*), 5 mg/ml (RF= 0.579, *p=< 0.05*), 10 mg/ml (RF=0.586, *p=< 0.05*) (Table 19.). *Daf-2* expression, when tested with sage was significantly increased at 1 mg/ml (RF=1.45, *p=<0.05*), 5 mg/ml (RF=1.42, *p=<0.05*), and 10 mg/ml (RF=1.58, *p=0.003*) relative to control (Table 19).

Table 19. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Sage								
	1mg/ml			5mg/ml			10mg/ml		
	RF	**SE**	***p value***	**RF**	**SE**	***p value***	**RF**	**SE**	***p value***
dbl-1	1.000	0.159	*0.286*	1.312	0.163	***< 0.05***	1.436	0.045	***0.029***
daf-9	1.467	0.042	***< 0.05***	1.378	0.026	***< 0.05***	1.520	0.029	***< 0.05***
daf-12	0.864	0.110	***< 0.05***	0.865	0.107	***< 0.05***	0.862	0.084	***0.005***
daf-7	0.880	0.031	***0.002***	0.579	0.007	***< 0.05***	0.586	0.028	***< 0.05***
daf-2	1.450	0.103	***<0.05***	1.418	0.051	***<0.05***	1.580	0.179	***0.003***

Sage was tested at 1mg/ml, 5mg/ml, and 10mg/ml concentrations expressed as RF± SE. N=8.

Discussion

TGF-β Signaling in Response to Sage

In *C. elegans* the TGF-β signaling pathway is regulated by its ligand *daf-7* (growth differentiation factor (GDF-11) in humans), and the levels of this change depending on the stress in the cell (Roberts et al., 2010). When *daf-7* levels are high it typically indicates low stress, and it binds to TGF-β thereby activating the signaling cascade resulting in the overall activation of *daf-9* which encodes a cytochrome P450 (CYP450) enzyme that increases cholesterol delivery to cells and causes cells to grow and differentiate (Prahlad & Morimoto, 2008). This increase in cholesterol delivery is

also independently regulated by IGF (Gerisch & Antebi, 2004). This suggests that IGF and TGF-β operate simultaneously parallel to each other, converging at *daf-9* (Mak & Ruvkun, 2004). When the cellular stress levels are elevated, *daf-7* levels typically go down. TGF-β mediated regulation of neuronal growth and development typically goes down (Baumeister et al., 2006).

In our result we found an overall decrease in *daf-7*, but an increase in *daf-9* expression. This suggests *daf-9* expression was predominantly being regulated via insulin like growth factor (IGF) signaling, which is corroborated with the previously reported increase in *daf-2* insulin/IGF-1 receptor expression (Chapter 1). As it has been shown that the *daf-2* and the *daf-4* type II TGF-β receptor control reproductive development in a cell sequential manner (Mak & Ruvkun, 2004). Increased expression and activation of the *daf-2* could increase the propagation of the insulin signaling cascade, possibly resulting in increased growth and biosynthetic metabolism (Rafalski & Burnet, 2008). Research has shown that *daf-9* is regulated by both *daf-2* and *daf-7* and functions upstream of *daf-12* (Mak & Ruvkun, 2004) (Figure 9). The gene *daf-12* encodes a nuclear receptor (vitamin D receptor) (Gerisch & Antebi, 2004). It is known that *daf-9*/CYP450 enzymes mediate steroid hormone synthesis, and because *daf-9* acts upstream of the nuclear receptor gene *daf-12* evidence suggest that *daf-9* mediates hormonal regulation of *daf-12* (Mak & Ruvkun, 2004) (Figure 9). According to our results the gene expression of *daf-9* was upregulated in response to sage treatments. We also observed a downregulation in *daf-12* expression, which coincides with previous reports of *daf-12* regulation by *daf-9* (Mak & Ruvkun, 2004).

Dbl-1is another BMG superfamily member as is *daf-7*, however *dbl-1* regulates innate immune response by the production of small antimicrobial peptides (Roberts et al., 2010) (Figure 9). *Dbl-1* and *daf-7* are ligands for the same type II TGF-β (*daf-4*) receptor complex, but regulate distinct biological outcomes (Roberts et al., 2010). In response to sage treatment we observed an overall upregulation of *dbl-1* in response to sage extract.

Conclusion

In response to sage treatment we saw two different effects on TGF-β signaling. We saw a downregulation in *daf-7* expression, but we also saw an upregulation in *dbl-1* expression. This suggests that there was an increased innate immune response as a result of sage treatment. However, the decrease in *daf-7* expression may suggest predominate neuroprotection may be conferred by insulin signaling and not by TGF-β signaling.

CHAPTER VII

SAGE DELAYS SYMPTOMS OF EXPERIMENTALLY INDUCED ALZHEIMER'S DISEASE IN *C. ELEGANS*

Introduction

Possible Links to Alzheimer's Disease

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by progressive decline in cognitive and motor function, and are fast becoming a major public health concern. Of all the different neurodegerative diseases AD is most prevalent (Perrin et al., 2009). Even though the exact mechanism behind the etiology and pathophysiology is not clearly understood it is believed that dysregulation in acetylcholine (ACh) mediated neurotransmission, processing of amyloid β peptides, and tau protein metabolism are thought to be involved (Armstrong, 2011; Moreira et al., 2009; Zhang et al., 2009).

It is suspected oxidative stress as well as nutrient metabolism play a role, specifically increase in oxidative stress as a result of mitochondrial dysfunction as well as increased insulin signaling mediated by various protein kinases are thought to be involved. Specific mechanisms at the neuronal level involve loss of ACh mediated signaling resulting in neuronal atrophy (Moreira et al., 2009). Therefore one possible

means of AD management is thought to be by increasing the levels of ACh at post synaptic clefts (Castro et al., 2009). Unlike other neurotransmitters which are selectivelytaken up by neurons ACh is degraded by the enzyme ACh esterase (AChE) without a mechanism of reuptake (Reed et al., 2010). Therefore AChE inhibitors are thought to play a role in the management of AD. The problem is AChE serves a broad range of developmental purposes, and thus proper dosing and delivery of inhibitors has been a drawback for treatment success (Paraoanu & Layer, 2008).

On the other hand accumulation of amyloid β peptides especially amyloid $\beta_{1\text{-}42}$ is also thought to cause neuronal cell death, it is unclear if these peptides cause oxidative stress or they a result of oxidative stress (Armstrong, 2011; Butterfield et al., 2002; Hardy, 2009; Müller et al, 2008; Shirwany et al., 2007) (Figure 10). Proper synapse function for synaptic plasticity, and learning is linked to the enzyme activity of α-secretase (sAPP-α), this enzyme cleaves amyloid precursor peptides (APP) (Mousavi & Hellstrom-Lindahl, 2009). It is proposed that improperly cleaved APP by the sAPP-β or sAPP-ϒ leads to extracellular accumulation and oligomerization of the toxic amyloid $\beta_{1\text{-}42}$ peptide (Mousavi & Hellstrom-Lindahl, 2009) (Figure 10). However, a recent clinical trial found that there was no significant difference between sAPPβ and sAPPα (Mulugeta et al., 2011). Regardless the cause amyloid $\beta_{1\text{-}42}$ accumulation or plaque is thought to block receptors for ACh (Snyder et al., 2005). Additionally, over excitation of the neurotransmitter receptor N-Methyl D-Aspartate (NMDA) by excess glutamate signaling can alter cation flux (Lipton, 2004). This results in elevated ROS levels which causes damage to synapse lipid membranes, activates microglia, and initiates apoptotic signaling (Lipton, 2004).

The intercellular protein tau is involved in microtubule formation important for structural integrity of the neuron (Samsonov et al., 2004). However, hyperphosporylation of tau via various kinases such as AKT/GSK-3, CDK-5, PKA, and PKC can destabilize the tubules, releasing tau (Voronkov et al., 2011). Free tau can then oligomerize forming intracellular neurofibrillary tangles, which eventually cause cell death (Yao Zhang, Tian, et al., 2009) (Figure 10). There are conflicting theories on AD causation and therefore confliction treatment approaches. However, the most common current view on AD pathology is attributed to plaques formed by accumulation of the toxic amyloid $\beta_{1\text{-}42}$ peptide and/or neurofibular tangles due to hyperphosphorylation of the microtubule associated tau protein (Armstrong, 2011).

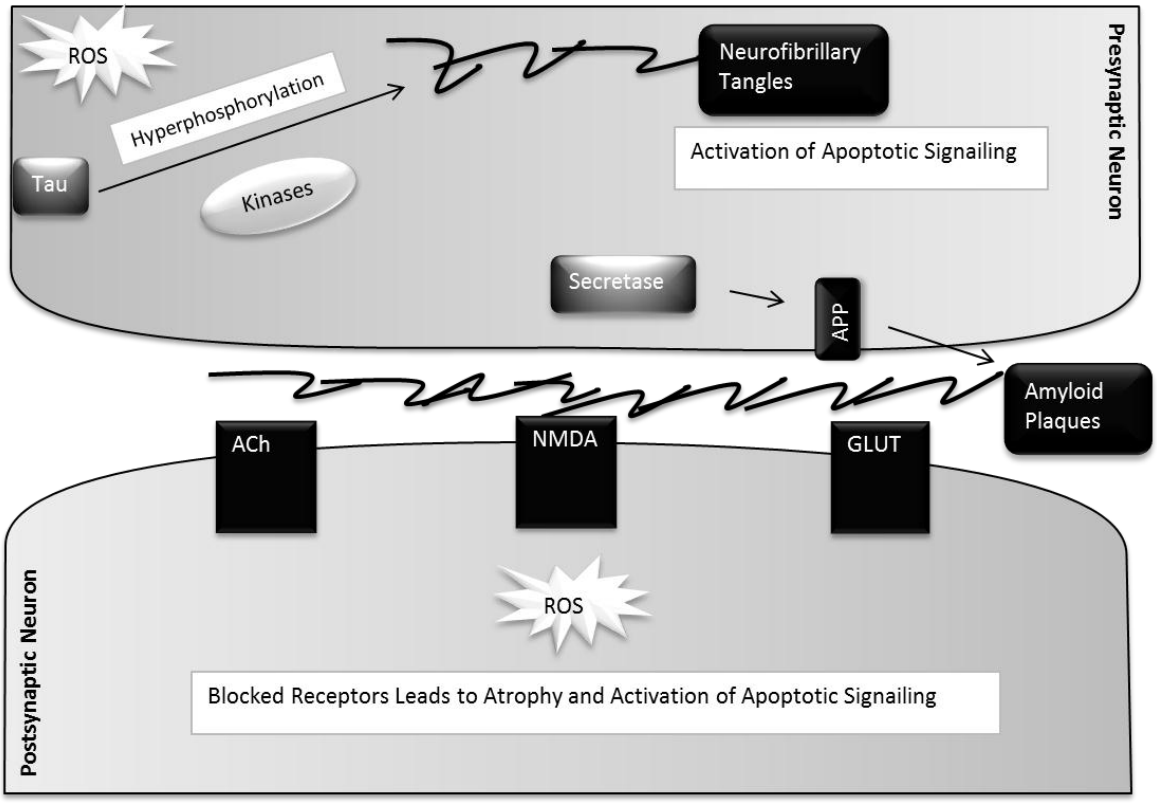

Figure 10. Possible molecular mechanisms behind development of Alzheimer's disease.

Natural Products and Sage for Management of Alzheimer's Disease

Extracts from plants such ginkgo biloba, and plant derived compounds like resveratrol, curcumin, and turmeric, have shown to have beneficial effects on various aspects of neurodegenerative processes including AD in both *in vitro* and *in vivo* models even though their mechanism is not completely understood (Iriti et al., 2010; Kannappan et al., 2011; Mattson & Cheng, 2006). As described earlier emerging evidence has also shown that *Salvia Officinalis* (sage) may have beneficial neuroprotecitve effects (EL-Kholy et al., 2010; Imanshahidi & Hosseinzadeh, 2006), evidence from *in vitro* models has suggested that it has antioxidant as well as anti-amyloid aggregation properties (Iuvone et al., 2006). Studies done in primary neuronal cultures has shown that compounds in sage can also inhibit anticholinesterase activities, therefore it may be promising for management of AD (Kennedy et al., 2006; Scholey et al., 2008). Despite this emerging research the effectiveness of sage in management of various symptoms of AD *in vivo* is not clearly understood. Therefore in this research we have decided to evaluate the effectiveness of sage at reducing amyloid β peptide induced paralysis in a novel *C. elegans* model. Furthermore we have tried to evaluate the nature of bioactive compounds in sage responsible for the anti-neurodegernative actions by using various fractions of sage in a *C. elegans* model. Unlike in a normal onset of AD where there is a progressive accumulation of amyloid β peptides and tau proteins, in the *C. elegans* model the accumulation of amyloid β is induced by thermal shock as described in the methods. Upon induction of amyloid β peptide expression the *C. elegans* progressively develop paralysis eventually resulting in total loss of mobility and death. In this model the delay

to the induction of paralysis after the thermal shock is used as a measure to determine the anti-neurodegenerative effects of sage.

Methods

Extraction and Fractionation of Sage

Aqueous extracts of sage were prepared by heating 1.5g of the herb in 30mL of distilled water at 60^{o}C for 30 minutes. The mixture was vacuum-filtered through Whatman filter paper, and filter sterilized. The aqueous extract (AE) was further fractionated into its polar acidic (PA), polar basic (PB) and polar neutral (PN) fractions using different solvents as shown in figure 11. These fractions were then evaluated in the neurodegeneration models.

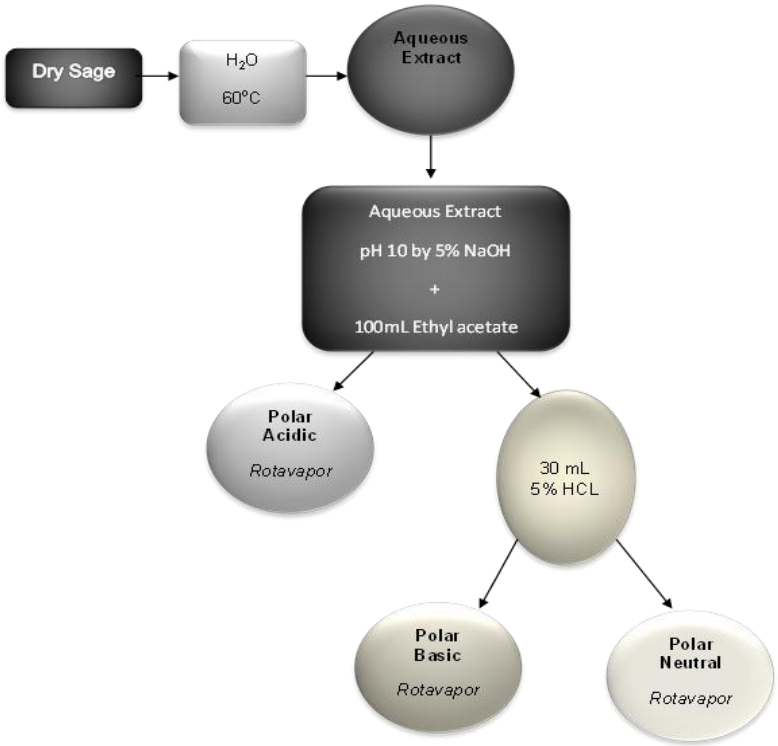

Figure 11. Method of solvent fractionation to collect samples of varying polarity and charge.

HPLC Analysis to Check Fractionation Efficiency

Different sage fractions were filtered through a 0.2 mm filter. 5 ml of sample was injected using Agilent ALS 1200 autosampler into Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with DAD 1200 diode array detector and a Agilent 1200 quarternary pump. The solvents used for gradient elution were (A) HPLC grade water with formic acid (0.1%) and (B) HPLC grade methanol with formic acid (0.1%). The methanol concentration was increased from 10% to 45% for the first 8 min and to 70% over the next 4 min, then decreased to 45% for the next 5 min and returned to 10% for the next 8 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C18, 250x4.6 mm i.d., with packing material of 5 mm particle size at a flow rate of 0.5 ml/min at ambient temperature. During each run the chromatogram was recorded at 210 nm, 254 nm, 280 nm and 300 nm and integrated using Agilent Chemstation enhanced integrator. As the HPLC is run in a reverse phase mode, the polar compounds are expected to have the shortest retention time, followed by neutral compounds and then basic compounds.

Nematode Propagation and Treatment

Transgenic *C. elegans* (CL4176) containing a heat-sensitive mutation developed to express human amyloid $\beta_{1\text{-}42}$ in the muscle tissue (Drake et al., 2003) were obtained from the *Caenorhabditis* Genetics Center. Many studies have indicated amyloid $\beta_{1\text{-}42}$ plays a role in AD. Propagation was conducted on 60 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1M CaCl, 1M MgSO4,

5 mg/mL Cholesterol in ethanol, 1M KPO4) at 16^{o}C (Brenner, 1974; Dostal & Link, 2010). 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 100 µl of *Escherichia coli* (*E. coli*) OP50 overnight cultures and incubated for 24 hours at 23^{o}C (Sutphin & Kaeberlein, 2009). The plates received a UV dose sufficient to arrest growth of the *E. coli* OP50 using a Stratagene UV Stratalinker 2400, which allowed for standardized *C. elegans* food supply (Sutphin & Kaeberlein, 2009).

Prior to the beginning of the experiment *C. elegans* were age synchronized. The adults from the synchronized population were allowed to lay eggs, and then the adults were removed. These worms were maintained at 16°C for until temperature up-shift to 25°C for initiation of amyloid β-induced paralysis (Dostal & Link, 2010). Mobility scoring was conducted beginning 20 hours after temperature up-shift and continued in 2 hour increments until all *C. elegans* are paralyzed (Dostal & Link, 2010). Worms were recorded as paralyzed when they were no longer able to conduct full body wave upon prodding, or when halos were formed around the head as paralysis was inevitable when only head movement was possible. Control plates did not contain any treatment whereas treatment plates contained aqueous extracts of the AE sage at 0.1% v/v, or one of the PA, PB or PN fractions. Extract and fractions were added to the NGM media just prior to pouring (Caldicott et al., 1994). *E. coli* OP50 mixed with the extract and fractions in the same test concentrations as the media, and treatment plates were inoculated and UV treated as previously described. Statistical analysis was modified from method in chapter 1.

Table 20. *C. elegans* transgenic gene to be evaluated and the human equivalent. Information obtained from www.wormbase.org.

Strain	Gene	Human Homolog	Wormbase-Gene ID
CL4176	***myo-3***	Amyloid β_{1-42}	WBGene00003515

Results

HPLC-ELSD-MS Analysis

HPLC profile analysis indicated that the fractionation protocol was effective as seen by figures 12-14. Different fractions exhibited different peaks and retention times the reverse phase C18 column. The PA fraction being more polar and negatively charged eluted the most compounds of the three fractions as indicated by the peaks in figure 12. The PB fraction contained compounds mostly positively charged, and resulted in fewer peaks as indicated by figure 13. PN compounds did not have a net charge and although resulted in more peaks than PB these eluted from the reverse phase C18 column later than either PA or PB fractions (Figure 14). Since we had three distinct retention time zones we can conclude that fractionation was effective.

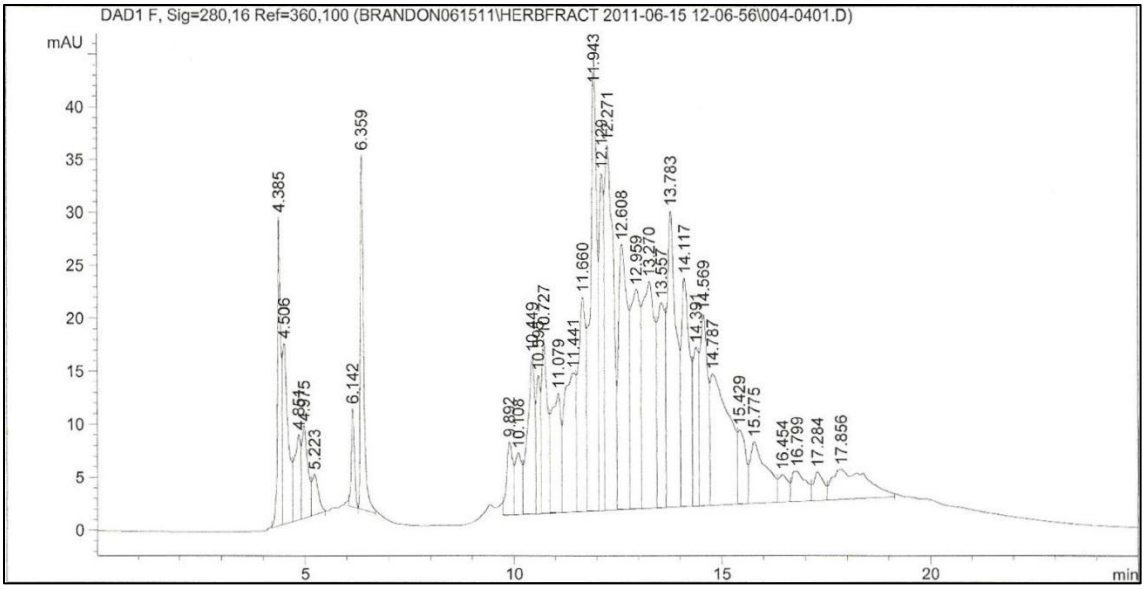

Figure 12. HPLC chromatogram for polar acidic (PA) sage fraction.

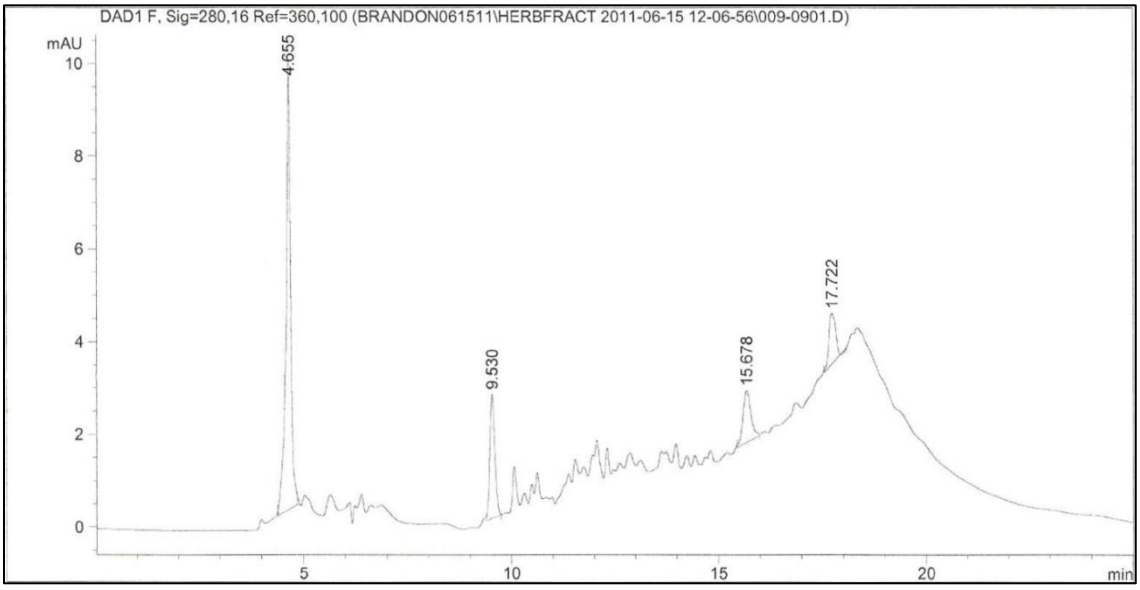

Figure 13. HPLC chromatogram for polar basic (PB) sage fraction.

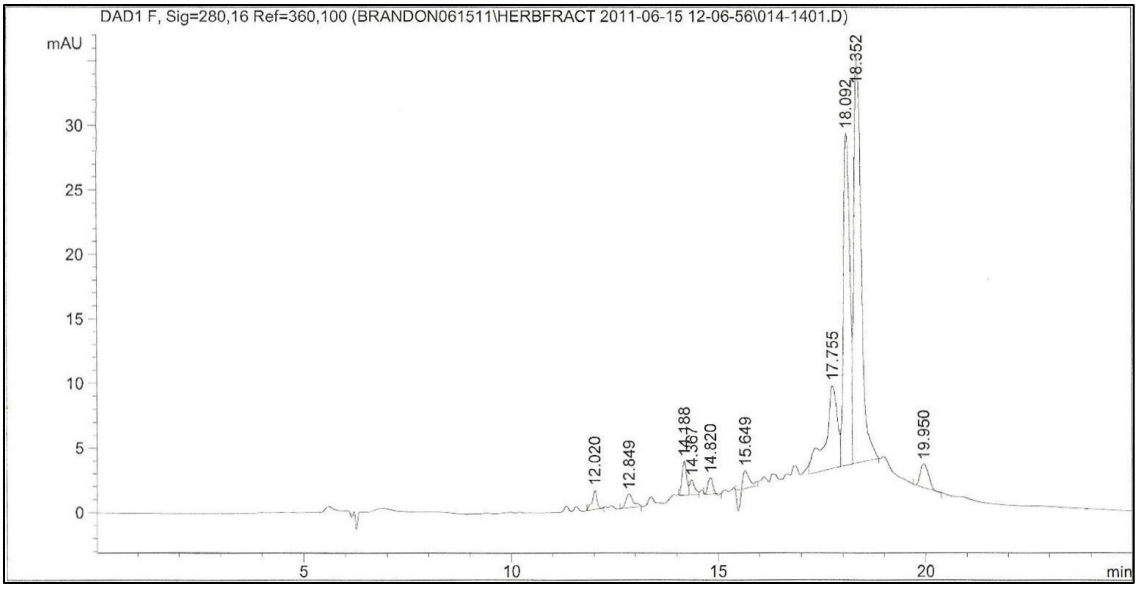

Figure 14. HPLC chromatogram for polar neutral (PN) sage fraction.

Effect of Sage in *C. elegans* Alzheimer's Neurodegeneration Model

As seen from figure 15 the time post heat shock to paralysis in 50% (PT_{50}) of worms on control plates was 26.52 hours. In worms that were incubated on plates with 0.1% v/v of AE the time to induction for PT_{50} was 27.48 hours, which was 57.6 minutes extended beyond control. The time to PT_{50} for PA treated animals was 30.05 hours, which was 3 hours and 31.8 minutes beyond control (Figure 16). For worms incubated on plates containing PB the time to PT_{50} was 28.69 hours, which was 2 hours and 10 minutes beyond control (Figure 17). While PT_{50} induction was at 27 hours for worms on PN plates, which was 28.8 minutes beyond control (Figure 18). Amongst all extracts and fractions tested the PA fraction was most effective in delaying induction of paralysis.

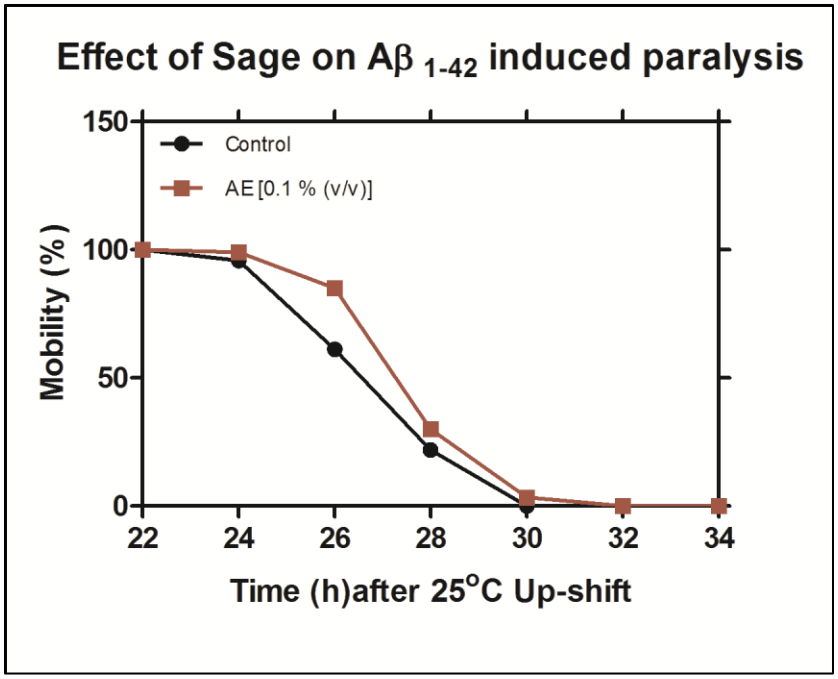

Figure 15. Mobility curve indicating percent of population mobile with AE treatment per time after 25°C temperature upshift.

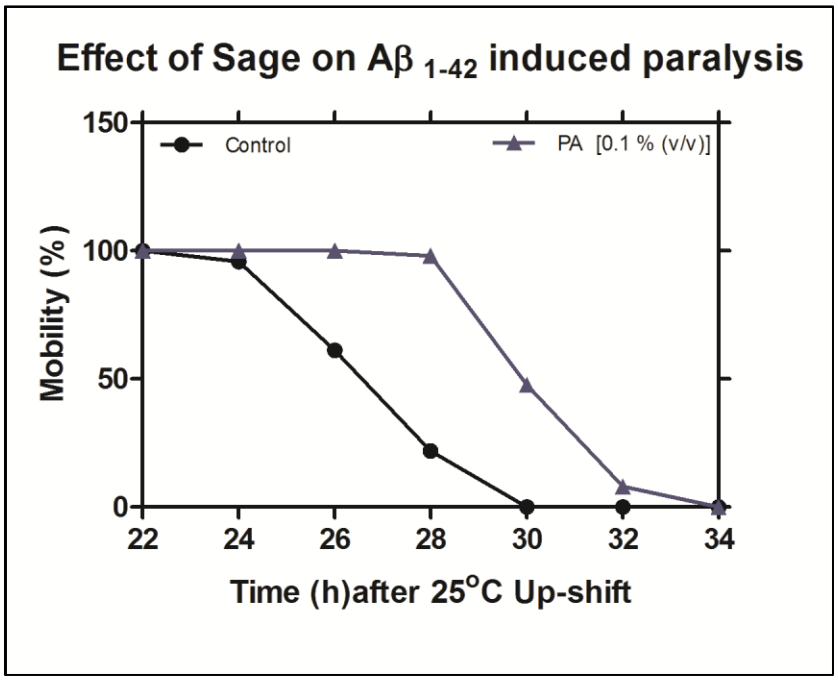

Figure 16. Mobility curve indicating percent of population mobile with PA treatment per time after 25°C temperature upshift**.**

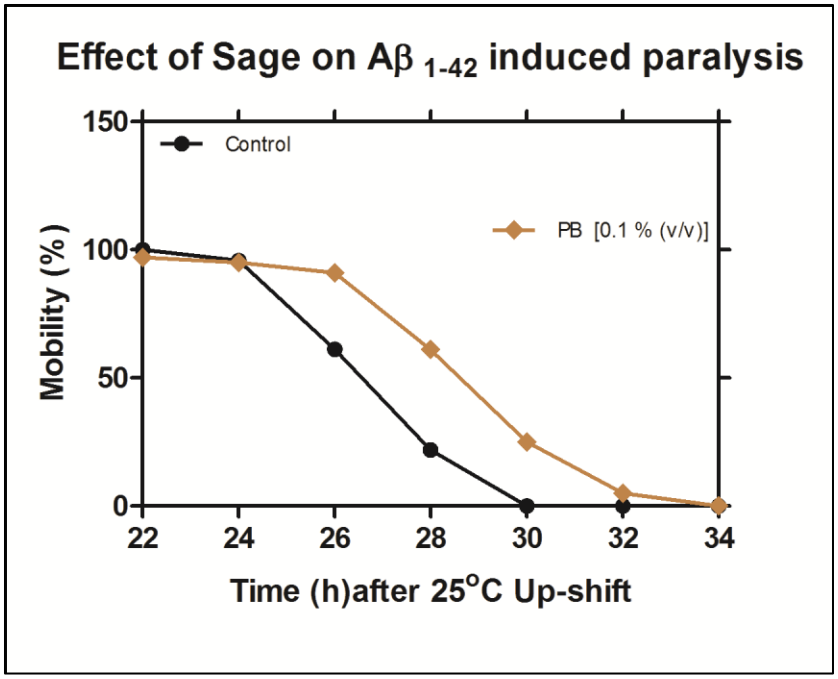

Figure 17. Mobility curve indicating percent of population mobile with PB treatment per time after 25°C temperature upshift.

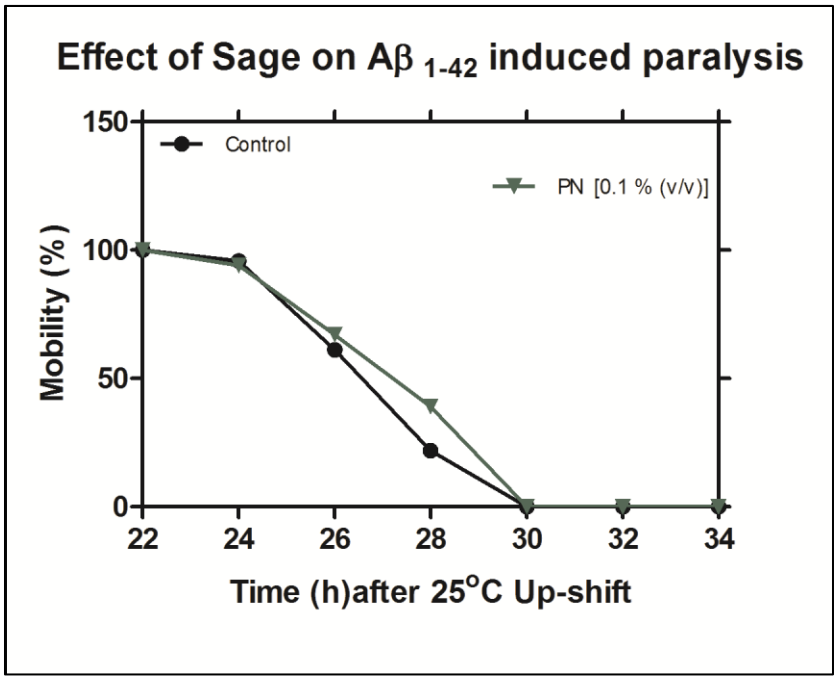

Figure 18. Mobility curve indicating percent of population moblie with PN treatment per time after 25°C temperature upshift**.**

Discussion

Effect of Sage on Paralysis in Alzheimer's Disease Model

There are many factors that have been implicated in the etiology of AD. We have studied major stress response pathways where dysregulation has been tied to AD such as ILS, Nrf-2/ARE, MAPK, HIF-1, Hsp/UPR, TGF-β. Dysregulation of any of these pathways has been shown to result in amyloid β plaques and/or tau neurofibrillary tangles resulting in activation of inflammatory and apoptotic signaling (Riviere et al., 2008). Since so many pathways are involved the most effective strategy for reducing these accumulations would be to address each of these pathways, not just a single pathway.

Studies have shown that the flavonoid polyphenols epicatechin and epicatechin gallate have a potent inhibitory effect on the aggregation of amyloid β_{1-40} and amyloid β_{1-42} peptides (Riviere et al., 2008). Small molecules, such as flavonoids have shown success in neurodegenerative disease models. Compounds that are contained in sage such as rosmarinic acid protected neuronal cells from amyloid β induced toxicity (Iuvone et al., 2006). It was also recently shown that betulinic acid promoted amyloid β fibril formation thereby reducing formation of the toxic soluble oligomers (Planchard et al., 2012). The compound ursolic acid was reported to inhibit amyloid β binding to receptor complexes thereby inhibiting the amyloid β binding to microglia (Wilkinson et al., 2011). Amyloid β bound microglia can induce proinflammatory cytokine and ROS production that are neurotoxic (Wilkinson et al., 2011). The phenolic compound caffeic acid was shown to reverse amyloid β toxicity by lowering intercellular Ca^{2+}, and phosphorylation of tau peptides (Sul et al., 2009). Chlorogenic acid has also shown to be neuroprotective

by anti-AChE, anti-amyloid β, and antioxidant actions (Kwon, Lee, et al., 2010). Since sage is known to contain all of these polyphenols (Mohsen Imanshahidi & Hosseinzadeh, 2006) it is possible that the delay in induction of paralysis in this *C. elegans* model is due to the beneficial effects of these dietary compounds either acting independently or in a synergistic manner. Our results also indicated that PA fraction of sage was most effective, this was similar to the effectiveness of caffeine as reported recently in the same model (Dostal & Link, 2010). Additionally, in previous chapters we have reported an increase in expression of *hsp16.2*, recent research studies have shown that constitutive expression of this Hsp may reduce the formation of amyloid β plaques in *C. elegans* (Fonte et al., 2008). This response may have reduced fibril formation until the point of over aggregation. Even though we did not specifically test tau phosphorylation mediated neurofibrillary tangle formation as reported earlier an increased expression of PP2A in response to sage treatments may also contribute to overall anti-neurodegnerative effects of sage. As it is well know that PP2A is an important regulatory enzyme in decreasing phosphorylation by MAP and other protein kinsases in the cell.

Conclusion

All of our treatments were effective in delaying the onset of paralysis in *C. elegans*. However, our results suggest that the PA compounds in sage may be more effective than other fractions. In our future experiments we will attempt to characterize the chemical nature of the PA fractions of sage, and further evaluate the neurodegenerative properties.

CHAPTER VIII

SALVIA OFFICINALIS AMELIORATES EXPERIMENTALLY INDUCED PARKINSON'S DISEASE IN *C. ELEGANS*

Introduction

Parkinson's Disease Signs and Symptoms

Parkinson's disease (PD) is a progressive neurodegenerative pathology characterized by degradation of dopaminergic neurons located primarily in the *substantia nigra pars compacta* (Jankovic, 2008). The most common presenting symptoms of PD are ridged posture, and uncontrolled shaking, which can be followed by dementia (Jankovic, 2008). Although genetics and environmental stressors are thought to play large roles in the pathophysiology, there are still many aspects of the disease that are not completely understood (Mazzio et al., 2011).

Dopamine Signaling

Dopamine has been discovered throughout the brain, but it tends to be in the highest concentrations in the corpus striatum portion of the *substantia niagra pars compacta* which makes up the basal ganglia, which is important for proper motor function (Matsumoto & Hikosaka, 2009). The catecholamine dopamine is synthesized from tyrosine by the enzyme tyrosine hydroxylase which converts it to L -3,4-

dihydroxyphenylalanine (levodopa or L-DOPA) (Mexas et al., 2011). L-DOPA is then acted on by L-DOPA decarboxylase to form dopamine (Kokkinou et al., 2009). This dopamine is then packaged into transport vesicles and then eventually secreted into the synaptic cleft by the monoamine transporters (González-Hernández et al., 2004). Upon release dopamine has three different fates. There is either degradation by monoamine oxidase (MAO), reuptake by dopamine transporter (DAT), or uptake by the post synaptic dopamine receptors D1 and D2 (Neve et al., 2004). The dopamine receptors function in a G- protein coupled receptor (GPCR) mediated manner (Neve et al., 2004). The binding of dopamine to a receptor results in the release of a G-protein from the GPCR (Beaulieu & Gainetdinov, 2011). When dopamine binds to D1 it results in the release of the domain GS, whereas when it binds to D2 it results in the release of the domain GI (Beaulieu & Gainetdinov, 2011). GS then binds to adenylylate cyclase causing its activation resulting in the release of cyclic AMP, and once cAMP is released it activates PKC and thereby propagates the dopamine signal (Wang, & Friedman, 2002). The release of GI also involves the binding of adenylate cyclase but causes its inactivation resulting in reduced levels of cytoplasmic cAMP which consequently does not activate PKC and therefore inhibits dopamine signaling (Wang, & Friedman, 2002). Dopamine via the D1 receptor results in contraction and movement of neuromuscular junctions, whereas dopamine via D2 results in inhibition of movement (Qu et al., 2008) (Figure 19).

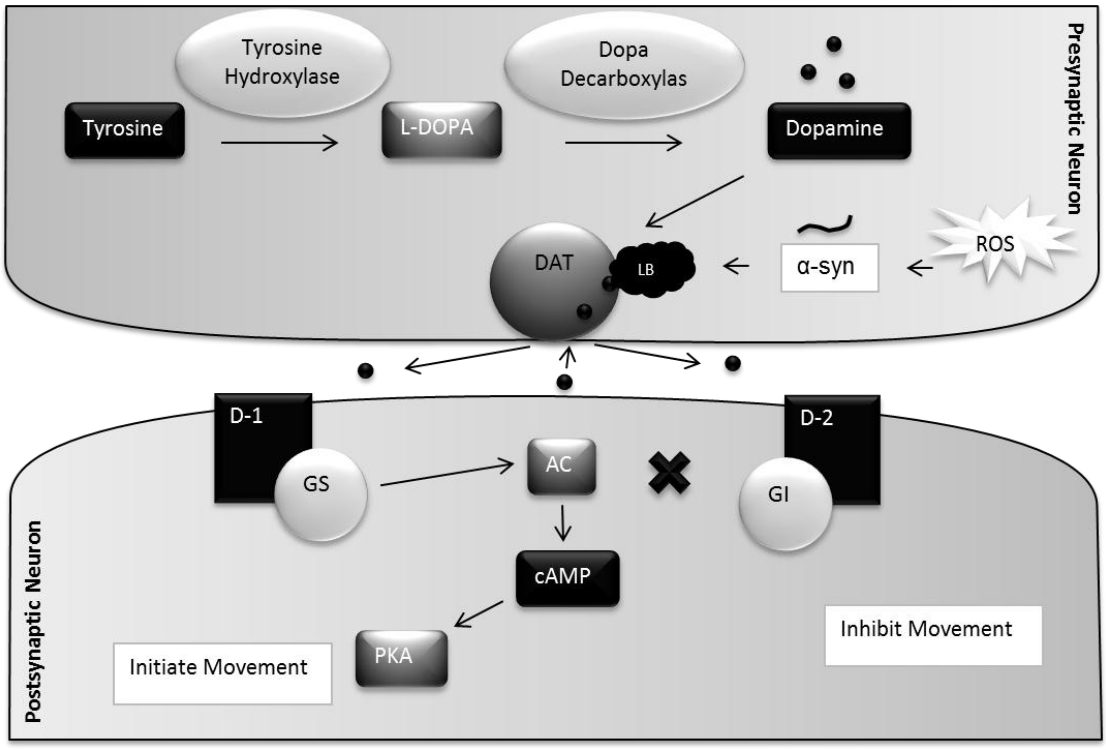

Figure 19. Dopamine synthesis, processing and transfer from presynaptic to postsynaptic neurons, and possible causes of Parkinson's disease development.

Possible Links to Parkinson's Disease

Studies using genetic models of PD have found that several familial mutations in genes can also result in PD like symptoms (Ogaki et al., 2011). Some of the most important are mutations in PTEN induced putative kinase 1 (PINK-1), and mutations in the protein α-synuclein. Mutations in α-synuclein leads to its accumulation and formation of Lewy bodies eventually causing apoptosis and neuronal cell death (Maraganore, 2011) (Figure 19). On the other hand PINK-1 mutation can result in decreased AKT signaling eventually causing apoptotic signaling and cell death (Gandhi et al., 2009). Additionally, emerging evidence also suggests that PD may be caused by environmental factors as well as exogenous toxins (Vibha et al., 2010). Though the exact mechanism by which

environmental factors and exogenous toxins cause PD is not well understood it is believed that prolonged exposure results in disruption of dopamine signaling, perhaps by effecting DAT-1 and the D1/D2 receptors (Braungart et al., 2004; Gandhi et al., 2009; Maraganore, 2011). It is also believed that environmental contaminates such as heavy metals and pesticides can increase mitochondrial oxidative stress and cellular protein stress (Tufekci et al., 2011). Both of these factors can lead to apoptotic signaling and progressively cause to cell death (Tufekci et al., 2011). Typically, in neurons misfolded proteins such as α-synuclein are targeted for proteasomal degradation by ubiquitination which is carried out by a protein known as parkin (Chin et al., 2011; Lin & Beal, 2006). A mutation or improper folding of either parkin or α-synuclein can result in accumulation of unusually high levels of α-synuclein which can lead to neuronal cell death (Mosharov et al., 2009). Additionally, chronic inflammation either resulting from oxidative stress or exogenous factors can activate certain protein and MAP kinase pathways which can also induce apoptosis in neuronal cells (Kim et al., 2010).

Current Parkinson's Disease Treatments

Several pharmaceutical strategies to manage PD symptoms have been developed over the years since causality is still not very well understood. Most of these treatments target methods to increase dopamine levels post secretion into the synaptic cleft. The most common and oldest method to manage the symptoms is to increase the levels of dopamine itself by pharmaceutically administering either dopamine or its precursor L-DOPA (Houghton & Howes, 2005). However this has several limitations pharmaceutical dopamine is not bioavailable as it can't cross the blood brain barrier (Pardridge, 2003). Therefore L-DOPA is administered, unfortunately the bioavailability of L-DOPA

deteriorates as the disease progresses (Boroojerdi et al., 2010). Moreover, it has recently been determined that natural sources of L-DOPA are better suited for increasing the levels of dopamine in the brain than compared to pharmaceutical sources (Morais & Almeida, 2003). This is primarily because of the presence of several other bioactive components that increase the absorption of L-DOPA through the blood brain barrier (Morais & Almeida, 2003). Other pharmaceutical therapies include either the inhibition of reuptake of dopamine via ingestion of DAT-1 inhibitors, or prevent the degradation of dopamine by MAOs (Robottom, 2011) . As both DAT-1 and MAOs are largely nonspecific this strategy results in large systemic side effects, which make the treatment risky (Nutt & Sexton, 2004; Xuan Wang et al., 2008). Therefore there is a constant search for compounds that can effectively manage PD. As described previously natural sources of dopamine have been extensively researched as a potential strategy for complementing pharmaceutical treatments. These include the beans *Mucuma pruriens* , and *Vicia faba* both of which synthesize L-DOPA (Morais & Almeida, 2003). However, because of relatively low levels of L-DOPA and difficulty of ingestion these products need to be administered at very high levels for a long period of time, and therefore have not been very successful.

Management of Parkinson's with Sage

Based on our previous results on the effect of sage on different stress response signaling pathways as well as its effectiveness at reducing some of the molecular mechanisms behind Alzheimer's disease we hypothesized that extracts of sage can also be effective in reducing some of the symptoms of exogenously induced PD in *C. elegans.* Here we have investigated the effect of AE sage extract and its fractions on 1-methyl-1-4-

phenylpyridium (MPP^+) induced dopamine neurodegeneration in various *C. elegans* models. Phenotypic changes of PD in *C. elegans* was quantified by scoring the induction of paralysis upon MPP^+ exposure, whereas changes in neurodegernation at the molecular level were evaluated by looking at the effect of sage treatments in the presence and absence of MPP^+ on critical genes involved in dopamine signaling (Braungart et al., 2004).

Methods

Nematode Propagation and Treatment

Transgenic strains of *C. elegans* with GFP promoter constructs of neuronal genes relevant to PD as well as wild type animals (Table 21.) were obtained from the *Caenorhabditis* Genetics Center. The neurotoxic molecule MPP^+ was used to institute the degradation of dopaminergic neurons creating symptoms similar to PD (Braungart et al., 2004). All strains were propagated as previously mentioned (Brenner, 1974). The strains of *C. elegans* were age synchronized. The adult *C. elegans* were removed which allowed for eggs to hatch, the L1 generation were washed by pipette with S-basal liquid media (0.59% NaCl, 5% 1M KPO4, 5 mg/ml Cholesterol in ethanol) (Lewis & Fleming, 1995) into a sterile 50 ml Falcon tube. Then S-complete [97.7% S-basal, 1% potassium citrate, 1% trace metals, 0.3% $CaCl_2$, 0.3% $MgSO_4$] liquid media was added to the tube with concentrated *E. coli* OP50 yielding a 5 mg/ml food supply (Lewis & Fleming, 1995). To each test well on a 96 well plate a total volume of 50 µl was added [10µl of IC_{90} MPP^+, 40 µl S-Complete (5 mg/ml *E. coli* OP50, and 17-23worms)].

Sage extracts and fractions were prepared as mentioned previously (Chapter 7). Additionally, treatment wells contained 0.1% v/v of the same AE, PA, PB, or PN sage treatments as used in the previous chapter (Chapter 7). The extract and fractions of sage were added to the S-complete at the selected concentration contributing to the 40 µl portion of well volume along with the *E. coli* OP50. Mature adult worms after timed egg lay were removed from maintenance plates, and after the eggs hatched the L1 generation was transferred to 96 well plates. These wells contained the extract or fraction treatment, S-complete, *E. coli* OP50, and IC_{90} dose of MPP^+ for a total well volume of 50 µl.

After 48 hours incubation the wild type (N2) worms were scored for mobility, worms were placed in three categories, wildtype movement, uncontrolled movement, or paralyzed. After 72 hours transgenic GFP expressing worms were imaged with Nikon SMZ1500 fluorescence microscope with Ri1/Qi1 CCD camera. The relative fluorescence with respect to MPP^+ only treated animals was then quantified using the National Institute of Health's ImageJ software (Iser & Wolkow, 2007). Statistical analysis was conducted as mentioned in chapter 1.

Table 21. List of *C. elegans* expressing transgenic neuronal genes to be evaluated by strain, and their human equivalents. Information obtained from www.wormbase.org.

Strain	Gene	Human Homolog	Wormbase-Gene ID
NL5901	***P unc-54***	Drives α-synuclien	WBGene00006789
BZ555	***dat-1***	Plasma membrane dopamine transporter	WBGene00000934
OH2411	***dop-1***	Dopamine receptor 1	WBGene00001052
OH1849	***dop-2***	Dopamine receptor 2	WBGene00001053
N2	**wildtype**	For mobility assay	N/A

Results

Effect of Sage on Mobility After 48h Exposure to MPP^+

The compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is actively metabolized by dopamine neurons to the toxic MPP^+ which selectively degrades these neurons due to excessive ROS production (Braungart et al., 2004). In wildtype (N2) *C. elegans* we evaluated AE, PA, PB, and PN sage extracts for capacity to delay the onset of paralysis. Animals were scored as paralyzed, or those displaying either wildetype or uncoordinated movement.

In control worms there was no induction of paralysis as there was no MPP^+ treatment. In worms that were exposed to MPP^+ 80% paralyzed after 48 hours of incubation. In worms that were exposed to MPP^+ but were cotreated with AE sage only 72% of the worms were scored as paralytic after 48 hours, indicating AE sage inhibited paralysis by 8%. In worms that were cotreated with PA sage and MPP^+ only 58% of the worms were paralyzed after 48 hours, suggesting that PA sage effectively inhibited paralysis by 22%. Worms that were exposed to MPP^+ and cotreated with PB sage were scored as 70% paralytic after 48 hours, thus we observed a 10% inhibition of paralysis. While worms exposed to MPP^+ but cotreated with PN sage were 77% paralytic at 48 hours, suggesting that PN sage only inhibited paralysis by 3%. Results represented in figure 20.

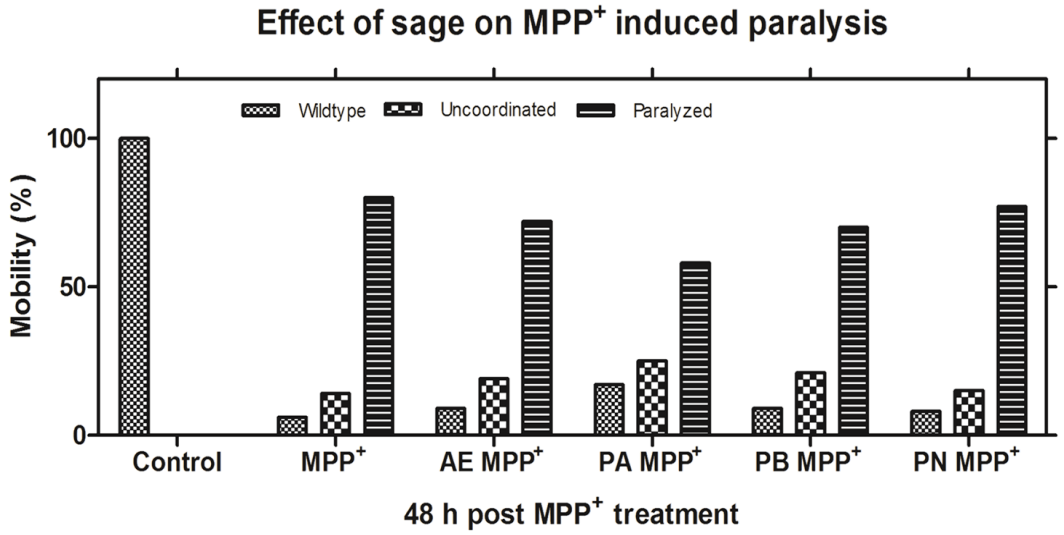

Figure 20. Mobility status of population represented by wildtype, uncoordinated, or paralyzed after 48 hours treatment with no MPP^+ (control), or MPP^+ with sage cotreatments.

Expression of Dopaminergic Genes in Response to Sage

We evaluated the response to sage extracts in genes important in dopamine signaling compared to the untreated control. In response to AE sage there was no significant difference in the expression of the gene *dop-1* (OH2411) from control. There was also no difference in *dop-1* expression in response to PA sage relative to control. When compared to control the *dop-1* expression was again not significantly different upon PB sage treatment. However, an upregulation was seen in *dop-1* expression in response to PN sage (RF=1.153, *p=0.038*) when compared to control. The gene expression of *dop-2* (OH1849) was upregulated in response to AE sage (RF=1.352, *p=0.013*), and PN sage (RF=1.519, *p=0.052*). While the expression of *dop-2* in response to PA sage and PB sage was no different from control. Expression of the gene *dat-1*

(BZ555) was significantly upregulated in response to AE sage (RF=1.610, *p=<0.05).* There was no significant difference in *dat-1* expression in response to PA and PB sage compared to control. We did however observe a downregulation in *dat-1* gene expression in response to PN sage (RF=0.771, *p=<0.05*) as compared to control. There was no significant change in *P unc-54* (NL5901) expression relative to control in response to AE sage, or any of it's the fractions.

Table 22. Dose dependent effect of sage treatments on fold-change in gene expression relative to control.

GENE	Response to Sage Compared to Control							
	AE		PA		PB		PN	
	RF	*p value*	RF	*p value*	RF	*p value*	RF	*p value*
dop-1	0.912	*0.228*	1.013	*0.210*	1.053	*0.317*	1.153	***0.038***
dop-2	1.352	*0.013*	0.951	*0.223*	1.051	*0.397*	1.519	***0.052***
dat-1	1.610	***<0.05***	0.975	*0.492*	0.929	*0.090*	0.771	***<0.05***
P unc-54	0.999	*0.402*	0.886	*0.128*	1.059	*0.122*	0.950	*0.097*

Sage extracts were tested at 0.1% (v/v) concentrations expressed as RF. N=6.

Expression of Dopaminergic Genes Exposed to MPP^+/Sage Cotreatment

The expression of *dop-1* was significantly downregulated upon exposure to MPP^+ (RF=0.645, *p=0.001*). However, *dop-1* expression was upregulated in worms cotreated with MPP^+/AE (RF=1.377, *p=0.056*) as compared to MPP^+ exposed worms that were not incubated with AE. A multifold upregulation was seen in *dop-1* expression when worms exposed to MPP^+ only were compared to MPP+/PA sage (RF=2.10, *p=0.003*) worms. An additional multifold upregulation in *dop-1* expression was seen in MPP^+/ PN sage (RF=2.527, *p=0.016*) worms, relative to worms exposed to MPP^+ only. Expression of *dop-1* in MPP^+/ PB sage exposed worms was not statistically different from worms

exposed to MPP^+ only. The expression of the gene *dop-2* was downregulated over control when exposed to MPP^+ (RF=0.585, *p=<0.05*). When compared to MPP^+ exposed worms, *dop-2* expression in MPP^+/AE cotreated worms was upregulated (RF=1.441, *p=0.005*). No significant change in *dop-2* expression compared to MPP^+ only worms was seen in MPP^+/PA sage worms. We observed increased *dop-2* expression in MPP^+/PB sage (RF=1.629, *p=0.004*) worms compared to those that were exposed to MPP^+ only. A downregulation in *dop-2* expression was seen in worms exposed to MPP^+/PN sage (RF=0.826, *p=0.032*) when compared to worms exposed to MPP^+ without PN cotreatment. The expression of the gene *dat-1* was downregulated when exposed to MPP^+ (RF=0.764, *p=0.033*). We observed an upregulation in *dat-1* expression upon AE sage (RF=1.577, *p=<0.05*) cotreatment with MPP^+ when compared to worms exposed to MPP^+ only. We also saw increased *dat-1* expression in MPP^+/PA sage (RF=1.150, *p<0.05*) worms compared to worms exposed to MPP^+ without PA cotreatment. An upregulation in *dat-1* gene expression was also seen in worms exposed to MPP^+/ PB (RF=1.357, *p=0.007*) as compared to worms exposed to MPP^+ exclusively. While *dat-1* expression was downregulated in MPP^+/PN sage (RF=0.857, *p=<0.05*) worms when compared to worms exposed to MPP^+ only. The expression of the gene *Punc-54* in the presence MPP^+ was not significantly different than control. The gene expression of *Punc-54* when exposed to MPP^+/AE sage was not significantly different from worms exposed to MPP^+ without AE. No change was observed in *Punc-54* expression for worms exposed to MPP^+ and PA sage compared to worms without PA sage cotreatment. There was also no statistical difference in *P unc-54* expression in PB sage/ MPP^+ worms from those that were not incubated with PB sage. Additionally, there was also no difference in *Punc-54*

gene expression for MPP^+/PN sage worms when compared to worms exposed to MPP^+ only.

Table 23. Dose dependent effect of sage cotreatments on fold-change in gene expression relative to worms exposed to MPP^+ only.

GENE	MPP^+ Cotreated With Sage Fractions									
	MPP^+		AE		PA		PB		PN	
	RF	*p* value	RF	*p value*	RF	*p value*	RF	*p value*	RF	*p value*
dop-1	0.645	***0.001***	1.377	***0.056***	2.010	***0.003***	2.055	*0.182*	2.527	***0.016***
dop-2	0.585	***<0.05***	1.441	***0.005***	1.002	*0.120*	1.629	***0.040***	0.826	***0.032***
dat-1	0.764	***0.033***	1.577	***<0.05***	1.150	***<0.05***	1.357	***0.007***	0.857	***<0.05***
P unc-54	0.980	*0.334*	0.998	*0.123*	0.871	*0.082*	1.028	*0.128*	0.977	*0.266*

Sage extracts and fractions were tested at 0.1% (v/v) concentrations expressed as RF. N=6.

Discussion

Effect of Sage on Chemically Induced Parkinson's Disease

As discussed previously MPTP induces neurodegneration in dopaminergic neurons because of its selective uptake by binding to the dopamine receptor and once inside the neurons it is metabolized to MPP^+ resulting in the creation of high amounts of ROS that causes neuronal cell death (Braungart et al., 2004). A possible mechanism by which sage extracts might be inhibiting MPP^+ induced changes in mobility is perhaps by the induction of pathways that result in management of the excessive oxidative stress. In our previous results we have shown that sage treatment upregulates the Nrf2/ARE signaling cascade, which as described is a main pathway for management of oxidative stress (Chapter 2). Research has also indicated that dysregulation of this pathway is important in neurodegeration and therefore its upregulation may provide protection against various neurodegenerative pathologies such as PD (Cook et al., 2011; Mazzio et

al., 2011; Tufekci et al., 2011; van Muiswinkel & Kuiperij, 2005). In addition, recent research with other natural products including *Ginkgo biloba* and the standardized extract EGb 761 have been effective *in vivo* on PD symptoms (Liang-Wei Chen et al., 2006). Similar results have also been seen with other natural products such as *Panax ginseng*, where it has been shown that reduction of ox stress in neuronal models can significantly reduce the MPTP induced degernation and therefore prevent PD (Liang-Wei Chen et al., 2006). Along with reducing ox stress which would eventually result in induction of apoptosis, sage extracts may also reduce neuronal death by inhibiting apoptotic signaling and inducing anti-apoptotic pathway, as also seen with *Ginkgo biloba* and *Panax ginseng* (Liang-Wei Chen et al., 2006). We have also discussed the careful balance between apoptotic and anti-apoptotic signaling in response to sage treatment in *C. elegans*. Here we evaluated phenotypic changes in mobility upon cotreatment with sage extract and fractions compared to MPP^+ in wild-type animals. We observed that all of the sage extracts were effective at reducing the MPP^+ induced paralysis. However, the most effective treatment was the PA fraction, followed closely by PB, AE, and PN sage.

When compared to untreated *C. elegans* the expression of *dop-1* decreased in response to MPP^+ suggesting a degradation of *dop-1* containing neurons. Significant restoration in green fluorescent protein (GFP) expression tagged to *dop-1* in MPP^+ treated worms that were cotreated with sage fractions suggests that there was less neurodegeneration in these worms. As expected the fluorescence of *dop-2* tagged GFP was also decreased in *C. elegans* neurons in response to MPP^+ suggesting a dopaminergic specific neuronal degeneration. However unlike with *dop-1* degeneration only the AE, and PB fraction were effective in significantly restoring *dop-2* GFP. Since proper

functioning of both of these receptors is essential for regulation of dopamine signaling our results indicate that the entire aqueous extract as well as the PB fraction may be the most effective treatment, this also possibly indicates a synergistic effect.

Similarly we also observed a significant degeneration of presynaptic neurons in which dopamine re-uptake occurs via *dat-1*. As MPP^+ is also recognized by *dat-1*once it is taken up by *dat-1* the result is cell death (Liang-Wei Chen et al., 2006). In response to sage treatment all of the extract of sage except the PN fraction resulted in a significant inhibition of MPP^+ induced degeneration in presynaptic neurons. This could either be due to induction of stress response signaling in the presynaptic neurons or because of the inhibition of MPP^+ binding to *dat-1* as seen previously with green tea polyphenols which were able to selectively inhibit MPP^+ reuptake in the synaptic cleft (Liang-Wei Chen et al., 2006).

Conclusion

Our results indicate *C. elegans* respond to MPP^+ and can effectively be used as ideal model to study chemically induced neurodegneration and PD like symptoms. We observed a significant decrease in mobility as a result of loss of dopaminergic signaling whereas cotreatments with PA and PB sage were most effective in reducing systemic MPP^+ induced paralysis. We hypothesized that this may be due to inhibition of MPP^+ induced apoptosis or by the induction of stress response signaling. In our previous studies we have shown that treatment with sage can upregulate oxidative stress response signaling as well as decrease the signaling of various apoptotic pathways. It is also possible that bioactive compound in sage may be directly interfering with MPP^+ binding

to dopamine receptors as well as transporters. Our previous studies have also shown that treatment with sage can increase GSH mediated detoxification pathways. Since the majority of the MPP^+ induced degeneration is thought to be caused by oxidative stress as a result of its phase I metabolic pathways it is likely that treatment with sage results in lower production of free radicals as a result of differential metabolism of MPP^+ via GSH phase II signaling. This was also clearly indicated by looking at the prevention of neuronal degradation of dopaminergic neurons with sage cotreatments.

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